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<p>(21) International Application Number: PCT/US96/04909  (22) International Filing Date: 5 April 1996 (05.04.96)  (30) Priority Data: 08/419,598 6 April 1995 (06.04.95) US  (71) Applicant (for all designated States except US): AMYLIN PHARMACEUTICALS, INC. [US/US]; 9373 Towne Centre Drive, San Diego, CA 92121 (US).  (72) Inventors; and (75) Inventors/Applicants (for US only): BEELEY, Nigel [GB/US]; 227 Loma Court Drive, Solana Beach, CA 92075 (US). RINK, Timothy, J. [GB/US]; 6041 Camino De La Costa, La Jolla, CA 92037 (US). ALBRANDT, Keith, Alan [US/US]; 5024 Via Playa Los Santos, San Diego, CA 92124 (US). SIERZEGA, Michael, Edward [US/US]; 3959 Eighth Avenue #6, San Diego, CA 92103 (US). JANES, Susan, M. [US/US]; 5676 Greenshade Road, San Diego, CA 92121 (US). PRICKETT, Kathryn, S. [US/US]; 7612 Trailbrush Terrace, San Diego, CA 92126 (US). PHELPS, Julie, L. [US/US]; 11264 Dalby Place, San Diego, CA 92126</p>	<p>(US). CHUN, Mark [US/US]; 8155 Cargill Avenue #23, San Diego, CA 92122 (US). PARK, Douglas, M. [US/US]; Apartment A, 10871 Via Los Narcisos, San Diego, CA 92129 (US). BEIDLER, Daniel, E. [US/US]; 12385 Paseo Colina, Poway, CA 92064 (US).  (74) Agents: DUFT, Bradford, J. et al.; Lyon &amp; Lyon, Suite 4700, 633 West Fifth Street, Los Angeles, CA 90071 (US).  (81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  Published With international search report.</p>	
<p>(54) Title: ANTI-OBESITY AGENTS</p> <p>(57) Abstract</p> <p>Proteins, including various dimeric and monomeric forms of the <i>ob</i> gene product, or modified <i>ob</i> protein-encoding DNA sequences, having anti-feeding activity and methods of preparing, purifying, formulating and using such proteins, for example, in the treatment of various feeding and metabolic disorders, including obesity and diabetes. Antibodies and immunoassays useful in the detection and quantitation of such proteins are also provided.</p>		

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## **ANTI-OBESITY AGENTS**

### **Related Applications**

5           This application is a continuation-in-part of United States Patent Application  
Serial No. 08/419,598, filed April 6, 1995, the contents of which are hereby  
incorporated in their entirety by reference.

### **Field of the Invention**

10           The present invention relates to specific proteins as well as recombinant  
versions of these proteins which have potent anti-feeding activity, to the preparation  
and purification and formulation of such proteins, to antibodies and immunoassays  
useful in their detection and quantitation, and to their use in methods for treating  
various feeding and metabolic disorders and conditions, including obesity and  
15   diabetes.

### **Background of the Invention**

It has been postulated that when humans overeat, the resulting extra fat  
somehow signals to the brain that the body is obese and makes it eat less and burn  
20   more fuel. The multi-decade search for such a signal is briefly outlined in Rink, In  
search of a satiety factor, (1994) *Nature*, 372: 406-407.

Identification of an *obese* (*ob*) gene from mice, the normal product of which  
is postulated to function as part of a signaling pathway from adipose tissue that acts  
to regulate the size of the body fat depot, was recently reported in Zhang et al.

Positional cloning of the mouse *obese* gene and its human homologue, (1994)

*Nature* 372: 425-432. Zhang et al. reports the cloning and sequencing of the mouse *ob* gene and its human homologue. According to the authors, *ob* encodes a 4.5-kilobase (kb) adipose tissue messenger RNA with a highly conserved 167-amino-acid open reading frame, the predicted amino-acid sequence of which is 84% identical between human and mouse and has features of a secreted protein.

Briefly, Zhang et al. relates the isolation and sequencing of 22 cDNA clones from a mouse white fat cDNA library. Zhang et al. at 428. According to this *Nature* report, the authors found a putative 97-base pair 5' leader followed by a predicted 167-amino-acid open reading frame and about a 3,700-kb 3'-untranslated sequence. Zhang et al. at 428. The *ob* cDNA sequence (reading from 5' → 3') and the predicted 167 amino acid sequence of the mouse *ob* gene product described in Zhang et al. are set forth below:

```
15  ATG TGC TGG AGA CCC CTG TGT CGG TTC CTG TGG CTT TGG TCC TAT
    M  C  W  R  P  L  C  R  F  L  W  L  W  S  Y
    15
    CTG TCT TAT GTT CAA GCA GTG CCT ATC CAG AAA GTC CAG GAT GAC
    L  S  Y  V  Q  A  V  P  I  Q  K  V  Q  D  D
    30
    20  ACC AAA ACC CTC ATC AAG ACC ATT GTC ACC AGG ATC AAT GAC ATT
        T  K  T  L  I  K  T  I  V  T  R  I  N  D  I
        45
        TCA CAC ACG CAG TCG GTA TCC GCC AAG CAG AGG GTC ACT GGC TTG
        S  H  T  Q  S  V  S  A  K  Q  R  V  T  G  L
    25  60
        ACT TTC ATT CCT GGG CTT CAC CCC ATT CTG AGT TTG TCC AAG ATG
```

D F I P G L H P I L S L S K M  
75  
GAC CAG ACT CTG GCA GTC TAT CAA CAG GTC CTC ACC AGC CTG CCT  
D Q T L A V Y Q Q V L T S L P  
5 90  
TCC CAA AAT GTG CTG CAG ATA GCC AAT GAC CTG GAG AAT CTC CGA  
S Q N V L Q I A N D L E N L R  
105  
GAC CTC CTC CAT CTG CTG GCC TTC TCC AAG ACD TGC TCC CTG CCT  
10 D L L H L L A F S K S C S L P  
120  
CAG ACC AGT GGC CTG CAG AAG CCA GAG AGC CTG GAT GGC GTC CTG  
Q T S G L Q K P E S L D G V L  
135  
15 GAA GCC TCA CTC TAC TCC ACA GAG GTG GTG GCT TTG AGC AGG CTG  
E A S L Y S T E V V A L S R L  
150  
CAG GGC TCT CTG CAG GAC ATT CTT CAA CAG TTG GAT GTT AGC CCT  
Q G S L Q D I L Q Q L D V S P  
20 165  
GAA TGC  
E C  
167

According to Zhang et al., computer analysis of the predicted protein sequence  
25 suggests the presence of an N-terminal signal sequence (underlined). Zhang et al. at  
428. The predicted signal sequence cleavage site is reported in Zhang et al. to be C-  
terminal to an alanine at position 21.

Zhang et al. also reports use of the coding sequence of the *ob* gene in  
hybridization experiments with genomic Southern blots of various vertebrate DNAs.

The article reports that such experiments yielded detectable signals in all vertebrate DNAs tested, from which it is concluded that *ob* is highly conserved among vertebrates. Zhang et al. at 429. To determine the extent of *ob* sequence conservation, Zhang et al. also reports on experiments to isolate and sequence cDNA clones that hybridize to *ob* from a human adipose tissue cDNA library. According to Zhang et al., the results of such experiments suggested that the nucleotide sequences from human and mouse were highly homologous in the predicted coding sequence, but had only 30% homology in the available 5' and 3' untranslated regions. Zhang et al. at 429. Alignment of the predicted human and mouse *ob* amino-acid sequences reported in Zhang et al. is set forth below:

```

10  Mouse MCWRPLCRFL WLWSYLSYVQ AVPIQKVQDD TKTLIKTIVT RINDISHTQS
    50
    Human MHWGTLGFL WLWPYLFYVQ AVPIQKVQDD TKTLIKTIVT RINDISHTQS
    Mouse VSAKQRTVL DFIPGLHPIL SLSKMDQTLA VYQQVLTSLP SQNVLQIAND
15  100
    Human VSSKQKVTGL DFIPGLHPIL TLSKMDQTLA VYQQILTSMP SRNVIQISND
    Mouse LENLRDLLHL LAFSKSCSLP QTSGLQKPES LDGVLEASLY
    STEVVALSRL 150
    Human LENLRDLLHV LAFSKSCHLP WASGLETLDL LGGVLEASGY
20  STEVVALSRL
    Mouse QGSLQDILQQ LDVSPEC
    167
    Human QGSLQDMLWQ LDLSPGC

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According to the authors, this alignment shows an 84% overall identity, and more extensive identity in the N-terminus of the mature protein, with only four conservative and three non-conservative substitutions among the residues between the presumed signal sequence cleavage site and the cysteine at position 117. Zhang

et al. at 430-431. As in mouse, states Zhang et al., 30% of the clones were missing the codon for glutamine at position 49.

In vitro translation of human ob RNA is also reported in Zhang et al. at 429. A human ob cDNA was said to have been subcloned by the authors into a pGEM cloning vector, and plus-strand RNA then synthesized using SP6 polymerase. Zhang et al. at 429. The in vitro synthesized RNA was translated in both the presence and absence of canine pancreatic microsomal membranes, the former revealing a single protein having an approximate molecular weight of 18 kD. Zhang et al. at 429. Zhang et al. reported that the addition of the microsomal membranes led to the appearance of a second translation product, also a single protein, having a molecular weight about 2 kD less than the primary translation product.

Although no evidence of functional activity was reported, based on their genetic investigations, Zhang et al. suggested that the ob gene product is a secreted, circulating factor that may represent at least one component of a satiety signaling system in the body, and that the level of expression of this gene may signal the size of the adipose depot. Thus, states Zhang et al., an increase in the level of the ob signal (for example, after a period of overeating) may act directly or indirectly on the central nervous system to inhibit food intake and/or regulate energy expenditure as part of a homeostatic mechanism to maintain constancy of the adipose mass. Zhang et al. at 431.

Obesity, excess adipose tissue, is becoming increasingly prevalent in developed societies. For example, approximately 30% of adults in the U.S. were estimated to be 20 percent above desirable body weight -- an accepted measure of obesity sufficient to impact a health risk (*Harrison's Principles of Internal Medicine*

12th Edition, McGraw Hill, Inc. (1991) p. 411). The pathogenesis of obesity is believed to be multifactorial but the basic problem is that in obese subjects food intake and energy expenditure do not come into balance until there is excess adipose tissue. Attempts to reduce food intake are usually fruitless in the medium term because the weight loss induced by dieting results in both increased appetite and decreased energy expenditure. Leibel et al., (1995) *New England Journal of Medicine* 322: 621-628. The intensity of physical exercise required to expend enough energy to materially lose adipose mass is too great for most people to undertake on a sufficiently frequent basis. Thus, obesity is currently a poorly treatable, chronic, essentially intractable metabolic disorder. Not only is obesity itself undesirable for social reasons, but obesity also carries serious risk of co-morbidities including, Type 2 diabetes, hypertension, atherosclerosis, degenerative arthritis, and increased incidence of complications of surgery involving general anesthesia. In those few subjects who do succeed in losing weight, by about 10 percent of body weight, there can be striking improvements in co-morbid conditions, most especially Type 2 diabetes in which dieting and weight loss are the primary therapeutic modality, albeit relatively ineffective in many patients for the reasons stated above.

Thus, it can be appreciated that an effective means to sustain weight loss is a major challenge and a superior method of treatment would be of great utility. Such a method, and compounds and compositions which are useful therefor, have been invented and are described and claimed herein.



### Summary of the Invention

The present invention is directed to the manufacture and use of dimeric forms of the *ob* gene product. We refer to these proteins as "*ob* dimers." Surprisingly, we have discovered that *ob* dimer proteins exhibit potent, prolonged inhibition of food intake in vivo. The invention is also directed to modified monomeric *ob* protein DNA sequences, to processes for the manufacture and purification of *ob* dimer proteins and *ob* monomer proteins, to formulations of *ob* dimers and *ob* monomer proteins, and to the use of these *ob* proteins and compositions in the treatment of subjects with disorders or conditions that would benefit from administration of these proteins and compositions.

In one aspect, then, the invention relates to *ob* dimers, which include human *ob* dimers, rat *ob* dimers, mouse *ob* dimers, as well as other vertebrate *ob* dimers. The invention also relates to *ob* dimer fusion proteins, including *ob* dimer fusion proteins that incorporate, for example, short marker or "reporter" peptides (for example, poly-histidine, and the eight amino acid marker peptide known in the art as "FLAG") which are useful in the detection and purification of the expressed protein, and are usually removable, but we have discovered in fact need not be removed in order to retain the activity of a non-fused *ob* dimer. We have also discovered that *ob* monomer fusion proteins, including human *ob* monomer fusion proteins, rat *ob* monomer fusion proteins, mouse *ob* monomer fusion proteins, as well as other vertebrate *ob* monomer fusion proteins, can be prepared as described above and elsewhere herein to yield in vivo appetite suppressant activity. According to this aspect of the present invention, there are provided substantially pure and pure *ob* dimers, *ob* fusion monomer proteins and *ob* fusion dimer proteins, which include

substantially pure and pure human *ob* dimers, human *ob* fusion monomer proteins and human *ob* fusion dimer proteins, substantially pure and pure rat *ob* dimers, rat *ob* fusion monomer proteins and rat *ob* fusion dimer proteins, and substantially pure and pure mouse *ob* dimers, mouse *ob* fusion monomer proteins and mouse *ob* fusion dimer proteins, as well as other substantially pure and pure vertebrate *ob* dimers, *ob* fusion monomers and *ob* fusion dimers. By "substantially pure" is meant purity in excess of about 50%, particularly at least about 80% by weight of protein. By "pure" is meant purity greater than or equal to about 90% and particularly greater than or equal to about 95% by weight of protein. Also described and claimed herein are preparations of pure, unfused *ob* monomer proteins.

In another aspect, the invention relates to recombinant methods of preparing and isolating *ob* dimers and *ob* dimer fusion proteins. One such method for the production of an *ob* dimer comprises the steps of (a) preparing a vertebrate cDNA library, preferably a vertebrate adipose cDNA library, and more preferably a human adipose cDNA library, (b) ligating said cDNA library into a cloning vector, (c) introducing said cloning vector containing said cDNA library into a first host cell, (d) contacting the cDNA molecules of said first host cell with a solution containing a suitable *ob* gene hybridization probe, (e) detecting a cDNA molecule which hybridizes to said probe, (f) isolating said cDNA molecule, (g) ligating the nucleic acid sequence of said cDNA molecule which encodes an *ob* protein into an expression vector, (h) transforming a second host cell with said expression vector containing said nucleic acid sequence of said cDNA molecule which encodes said *ob* protein, (i) culturing the transformed second host cell under conditions that favor the

production of said *ob* protein as a dimer, and (j) isolating said *ob* protein expressed by said second host cell.

In still another aspect, the invention relates to recombinant methods for the production of *ob* dimers which do not include the steps of making and screening a cDNA library or libraries, but use instead a method of amplification of cDNA prepared from tissue total RNA, preferably adipose tissue total RNA. Such a method is described herein and comprises the steps of (a) isolating a preparation of total RNA from a vertebrate tissue, preferably adipose tissue, (b) converting said isolated RNA to cDNA, (c) amplifying a cDNA sequence from said cDNA using oligonucleotide primers suitable for annealing to a target *ob* protein gene sequence, (d) detecting a cDNA molecule using oligonucleotides suitable for hybridization to said target *ob* protein gene sequence, (e) isolating said cDNA molecule, (f) ligating the nucleic acid sequence of said cDNA molecule which encodes an *ob* protein into an expression vector, (g) transforming a host cell with said expression vector containing said nucleic acid sequence of said cDNA molecule which encodes said *ob* protein, (h) culturing the transformed host cell under conditions that favor the production of said *ob* protein as a dimer, and (i) isolating said *ob* protein expressed by said host cell. In a variation of this method, tissue poly-A<sup>+</sup> RNA, preferably adipose tissue poly-A<sup>+</sup> RNA, is used in place of tissue total RNA or adipose tissue total RNA.

Another method of producing an *ob* dimer comprises the steps of (a) culturing a transformed host cell containing a DNA sequence encoding a vertebrate *ob* protein, preferably a human *ob* protein, under conditions that favor the production

of said vertebrate *ob* protein as a dimer, and (b) isolating said *ob* dimer expressed by said transformed host cell.

In any of the above methods of *ob* dimer production, any portions of the transformed host cell isolate that may be found to contain *ob* monomer may  
5 optionally be converted to *ob* dimer as described herein.

Still another method of producing an *ob* dimer comprises the steps of (a) culturing a transformed host cell containing a DNA sequence encoding a vertebrate *ob* protein, preferably a human *ob* protein, under conditions that favor the production of said vertebrate *ob* protein as a monomer, (b) isolating said *ob* protein expressed  
10 by said transformed host cell, and (c) dimerizing said *ob* protein by subsequent chemical transformation.

In another aspect, the invention relates to the production of *ob* dimer fusion proteins using recombinant methods. One such method for the production of an *ob* dimer fusion protein comprises the steps of (a) preparing a vertebrate cDNA library,  
15 preferably a vertebrate adipose cDNA library, and more preferably a human adipose cDNA library, (b) ligating said cDNA library into a cloning vector, (c) introducing said cloning vector containing said cDNA library into a first host cell, (d) contacting the cDNA molecules of said first host cell with a solution containing a suitable *ob* gene hybridization probe, (e) detecting a cDNA molecule which hybridizes to said  
20 probe, (f) isolating said cDNA molecule, (g) ligating the nucleic acid sequence of said cDNA molecule which encodes an *ob* protein to a second DNA sequence, preferably a marker DNA sequence encoding the FLAG peptide, to create a fusion DNA sequence, (h) ligating said fusion DNA sequence into an expression vector, (i) transforming a second host cell with said expression vector containing said fusion

DNA sequence, (j) culturing the transformed second host cell under conditions that favor the production of said *ob* fusion protein as a dimer, and (k) isolating said *ob* protein expressed by said second host cell.

*Ob* dimer fusion proteins may also be prepared by recombinant methods

5 which do not include the steps of making and screening a cDNA library or libraries, but use instead a technique involving the amplification of cDNA prepared from, for example, tissue total RNA, preferably adipose tissue total RNA. Such a method comprises the steps of (a) isolating a preparation of total RNA from a vertebrate tissue, preferably adipose tissue, (b) converting said isolated RNA to cDNA, (c)

10 amplifying a cDNA sequence from said cDNA using oligonucleotide primers suitable for annealing to a target *ob* protein gene sequence, (d) detecting a cDNA molecule using oligonucleotides suitable for hybridization to said target *ob* protein gene sequence, (e) isolating said cDNA molecule, (f) ligating the nucleic acid sequence of said cDNA molecule which encodes an *ob* protein to a second DNA

15 sequence to create a fusion DNA sequence encoding an *ob* fusion protein, (g) ligating said fusion DNA sequence into an expression vector, (h) transforming a host cell with said expression vector containing said fusion DNA sequence, (i) culturing the transformed host cell under conditions that favor the production of said *ob* fusion protein as a dimer, and (j) isolating said *ob* dimer fusion protein expressed by said

20 host cell. In a variation of this method, tissue poly-A<sup>+</sup> RNA, preferably adipose tissue poly-A<sup>+</sup> RNA, is used in place of tissue total RNA or adipose tissue total RNA.

Another method of producing a recombinant *ob* dimer fusion protein comprises the steps of (a) culturing a transformed host cell containing a DNA

sequence encoding a vertebrate *ob* protein, preferably a human *ob* protein, coupled to a marker DNA sequence, preferably a marker DNA sequence encoding the FLAG peptide, under conditions that favor the production of said vertebrate *ob* fusion protein as a dimer, and (b) isolating said *ob* dimer fusion protein expressed by said  
5 transformed host cell.

In any of the above methods of *ob* dimer fusion protein production, any portions of the transformed host cell isolate that may be found to contain *ob* fusion monomer may optionally be converted to *ob* dimer fusion protein as described herein.

10 Still another method of producing a recombinant *ob* dimer fusion protein comprises the steps of (a) culturing a transformed host cell containing a DNA sequence encoding a vertebrate *ob* protein, preferably a human *ob* protein, coupled to a marker DNA sequence, preferably a marker DNA sequence encoding the FLAG peptide, under conditions that favor the production of said vertebrate *ob* fusion  
15 protein as a monomer, (b) isolating said *ob* fusion protein expressed by said transformed host cell, and (c) dimerizing said *ob* fusion protein.

In yet another aspect, the invention relates to improved methods for the periplasmic expression and purification of *ob* dimer protein, *ob* dimer fusion protein, *ob* monomer protein, and *ob* fusion monomer protein, which provide increased  
20 protein yield and quality. These methods include the use of a T7 promoter vector construct transfected into *E. coli* BL21(DE3) cells which are grown at about 25° to about 30°C in media containing a supplemental carbon source, preferably glucose, for enhanced expression. Preferred purification methods include the use of an osmotic shock protocol which incorporates one or more specific protease inhibitors,

preferably Peflabloc SC, followed by the addition of BisTris-propane, or buffers of a similar nature, and separation using a cellulose-based anion exchange chromatography resin, preferably DE-52 resin. Further purification may be undertaken using high pressure liquid chromatography, preferably reversed phase  
5 high pressure liquid chromatography.

We have also invented improved methods for the intracellular expression (into inclusion bodies) of recombinant *ob* proteins, and their subsequent solubilization, refolding and purification, which provide greatly increased protein yield in *E. coli*. In this method, certain naturally-occurring nucleotides within the  
10 codons for Val<sup>22</sup> and Pro<sup>23</sup> in the coding sequences for any of the mammalian *ob* proteins, including human, rat and mouse, are replaced. Solubilization, refolding and purification of the recombinant proteins are accomplished by lysing the cells and washing inclusion bodies in an anionic buffer of approximately neutral pH, preferably 100mM phosphate at a pH of about 6.5, dissolving the inclusion bodies in  
15 a buffer containing a chaotropic agent, such as urea in ammonium bicarbonate buffer, transferring the protein by dialysis or dilution into BisTris-propane or a similar buffer, and purifying the protein using a cellulose-based anion exchange chromatography resin, preferably DE-52 resin. The invention also provides for the preparation of *ob* monomer protein or *ob* monomer fusion protein without unwanted  
20 dimer formation by the addition of agents such as glutathione and dithiothreitol to any or all of the above-noted buffers. Further purification may be undertaken using high pressure liquid chromatography, preferably reversed phase high pressure liquid chromatography.

In still another aspect, the invention provides for the chemical synthesis, using solid phase peptide synthesis or a combination of both solid phase peptide synthesis and solution chemistries, as a further method of preparation of the *ob* proteins of the invention. Following chemical synthesis and isolation of the  
5 resulting product, for example, by high pressure liquid chromatography, cation and/or anion exchange chromatography methods, dimerization may be achieved by incubation in an appropriate buffer at a dimer-formation-enhancing pH, preferably a pH of from 7 to 9, or by differential protection and selective deprotection of the cysteine residues.

10 In a still further aspect, the invention provides *ob* dimer and *ob* monomer compounds and pharmaceutical compositions, and methods for the treatment of disorders and conditions which may benefit from the administration of such compounds and compositions, including obesity and diabetes, particularly Type 2 diabetes. Such pharmaceutical compositions include therapeutically effective  
15 amounts of an *ob* dimer or *ob* dimer fusion protein or *ob* monomer protein or *ob* monomer fusion protein in pharmaceutically acceptable carriers. *Ob* dimers and *ob* dimer fusion proteins include human *ob* dimers and human *ob* dimer fusion proteins, rat *ob* dimers and rat *ob* dimer fusion proteins, mouse *ob* dimers and mouse *ob* dimer fusion proteins, as well as other vertebrate *ob* dimers and vertebrate *ob* dimer fusion  
20 proteins. *Ob* monomer proteins and *ob* monomer fusion proteins include human *ob* monomer and *ob* monomer fusion proteins, rat *ob* monomer and *ob* monomer fusion proteins, mouse *ob* monomer and *ob* monomer fusion proteins, as well as other vertebrate *ob* monomer and *ob* monomer fusion proteins. Preferably, these *ob* dimer and *ob* monomer compounds are prepared in a stable lyophilized form as



trifluoroacetate, acetate, hydrochloride, or ammonium bicarbonate salts, most preferably ammonium bicarbonate salts. Preferred stable solutions of these *ob* dimer and *ob* monomer compounds are prepared using BisTris-propane, or buffers of similar structure, with or without a detergent, such as Tween 80, to have a pH from  
5 about 7.5 to about 9, and most preferably a pH of about 8.5.

Treatment methods comprise the administration of a therapeutically effective amount of a pharmaceutical composition comprising an *ob* dimer and/or *ob* monomer compound of the invention to patients in need thereof. Such patients include obese and diabetic patients (particularly Type 2 diabetic subjects), and others  
10 whose condition would benefit from administration of an *ob* dimer or *ob* monomer protein in an amount useful to promote reduced food intake or increased energy expenditure or both.

Pharmaceutical *ob* dimer compositions and *ob* monomer protein compositions may be administered separately or together with other compounds and  
15 compositions that exhibit a short-term satiety action including but not limited to other compounds and compositions that comprise an amylin or an amylin agonist. Suitable amylin agonists include, for example, human amylin and rat amylin. Suitable amylin agonists include, for example, [Pro<sup>25,28,29</sup>]-human amylin and salmon calcitonin.

20 In still another aspect, the present invention provides novel antibodies, including polyclonal antibodies, preferably monoclonal antibodies, and antibody fragments which can be produced in mice or by recombinant cell lines or by hybrid cell lines, the antibodies being characterized in that they have certain predetermined specificity to *ob* dimers, *ob* dimer fusion proteins, and *ob* monomer fusion proteins

over their corresponding *ob* monomers. By virtue of their specificity, such antibodies and antibody fragments are useful in methods for the purification of *ob* dimers, *ob* dimer fusion proteins, and *ob* monomer fusion proteins, and in the immunoassay of these target antigens.

5

### Definitions

The term "amino acid" refers to the natural L-amino acids. Natural L-amino acids include alanine (Ala or A), arginine (Arg or R), asparagine (Asn or N), aspartic acid (Asp or D), cysteine (Cys or C), glutamine (Gln or Q), glutamic acid (Glu or E),  
10 glycine (Gly or G), histidine (His or H), isoleucine (Ile or I), leucine (Leu or L), lysine (Lys or K), methionine (Met or M), phenylalanine (Phe or F), proline (Pro or P), serine (Ser or S), threonine (Thr or T), tryptophan (Trp or W), tyrosine (Tyr or Y), and valine (Val or V).

The term "peptide" refers to a sequence of amino acids linked predominantly  
15 though not necessarily exclusively through their alpha-amino and carboxylate groups by peptide bonds. Such sequences as shown herein are presented in the amino (N terminus) to carboxy (C terminus) direction, from left to right.

The term "protein" refers to a molecule comprised of one or more peptides.

The term "cDNA" refers to complementary deoxyribonucleic acid.

20 The term "nucleic acid" refers to polymers in which bases (*e.g.*, purines or pyrimidines) are attached to a sugar phosphate backbone. Nucleic acids include DNA and RNA.

The term "mRNA" refers to messenger ribonucleic acid.

The term "nucleic acid sequence" refers to the sequence of nucleosides comprising a nucleic acid. Such sequences as shown herein are presented in the 5' to 3' direction, from left to right.

The term "recombinant" refers to a DNA molecule comprising pieces of  
5 DNA that are not normally contiguous, or to a protein expressed therefrom.

### **Brief Description of the Drawings**

**FIGURE 1** shows the nucleotide and deduced amino acid sequences of the rat *ob* gene.

10 **FIGURE 2** shows the change in food intake in *ob/ob* mice administered various doses of rat *ob* protein.

**FIGURE 3** shows the change in food intake in *ob/ob* mice administered various doses of rat *ob* protein and two doses of Met-rat *ob* protein.

15 **FIGURE 4** shows the change in food intake in *ob/ob* mice administered various doses of rat *ob* protein and three doses of Met-rat *ob* dimer protein.

**FIGURE 5** shows the change in food intake in *ob/ob* mice administered various doses of rat *ob* protein and two doses of FLAG-rat *ob* protein.

**FIGURE 6** shows the change in food intake in *ob/ob* mice administered various doses of rat *ob* protein and two doses of FLAG-rat *ob* dimer protein.

20 **FIGURE 7** shows the change in food intake in *NIH/Sw* mice administered various doses of rat *ob* protein.

**FIGURE 8** shows the change in body weight in *ob/ob* mice administered various doses of rat *ob* protein.

**FIGURE 9** shows the change in body weight in *ob/ob* mice administered various doses of rat *ob* protein and two doses of Met-rat *ob* protein.

**FIGURE 10** shows the change in body weight in *ob/ob* mice administered various doses of rat *ob* protein and three doses of Met-rat *ob* dimer protein.

5       **FIGURE 11** shows the change in body weight in *ob/ob* mice administered various doses of rat *ob* protein and two doses of FLAG-rat *ob* protein.

**FIGURE 12** shows the change in body weight in *ob/ob* mice administered various doses of rat *ob* protein and two doses of FLAG-rat *ob* dimer protein.

**FIGURE 13** shows the change in body weight in *NIH/Sw* mice administered  
10       various doses of rat *ob* protein.

### **Detailed Description of the Invention**

The present invention is directed to dimeric forms of the *ob* gene product, including *ob* dimers and *ob* dimer fusion proteins, as well as to *ob* monomer fusion  
15       proteins, and to the manufacture and use of such compounds. The invention is also directed to modified monomeric *ob* protein DNA sequences, to processes for the recombinant expression and purification of *ob* dimer proteins and *ob* monomer proteins in high yield, to formulations of these *ob* dimers and *ob* monomer proteins, and to the use of these *ob* proteins and compositions in the treatment of subjects with  
20       disorders or conditions that would benefit from administration of these proteins and compositions, including but not limited to those subjects who would benefit from reduced food intake or increased energy expenditure or both.

*Ob* dimers include dimers of the *ob* gene product from any vertebrate source, including but not limited to human, mouse, and rat. An example of a rat *ob* gene

sequence useful in preparing an *ob* dimer or other *ob* product is described herein and depicted in Figure 1. An especially preferred rat *ob* DNA sequence for use in the preparation of the compounds and compositions of the invention and which is described and claimed herein includes GTT as the codon for Val<sup>22</sup> (in place of the naturally-occurring GTG codon) and CCG as the codon for Pro<sup>23</sup> (in place of the naturally-occurring CCT codon).

Examples of human and mouse *ob* genes useful in preparing these dimers and other *ob* products of the invention are found in Zhang et al., Positional cloning of the mouse obese gene and its human homologue, (1994) *Nature* 372: 425-432.

This article, and all other publications referenced herein, are hereby incorporated in their entirety by reference. An especially preferred human *ob* DNA sequence for use in the preparation of the compounds and compositions of the invention and which is described and claimed herein includes GTT as the codon for Val<sup>22</sup> (in place of the naturally-occurring GTG codon) and CCG as the codon for Pro<sup>23</sup> (in place of the naturally-occurring CCC codon). Optionally, the human *ob* DNA sequence also contains CAG as the codon for Gln<sup>25</sup> (in place of the naturally-occurring codon CAA). An especially preferred mouse *ob* DNA sequence includes GTT as the codon for Val<sup>22</sup> (in place of the naturally-occurring GTG codon) and CCG as the codon for Pro<sup>23</sup> (in place of the naturally-occurring CCT codon). Other vertebrate *ob* sequences may be isolated in accordance with the methods described herein for use in the preparation of their corresponding dimers, dimer fusion proteins, monomers and monomer fusion proteins.

The *ob* dimers and *ob* dimer fusion proteins of the present invention, as well as their corresponding *ob* monomers and *ob* fusion monomer proteins, include those

having variations in a known or disclosed *ob* gene sequence or sequences, including fragments, naturally occurring mutations, allelic variants, randomly generated artificial mutants and intentional sequence variations (including proteins having an N-terminal methionine residue), and corresponding protein alterations, all of which

5 conserve relevant *ob* protein activity, for example, anti-feeding activity. An example of such a variation is the microheterogeneity of the cDNA in the human and mouse *ob* dimer gene sequences where about 70% of the cDNAs have a glutamine codon at position 49 and about 30 % do not. The term "fragments" refers to any part

10 of the *ob* sequence which contains fewer amino acids than the complete protein, as for example, partial sequences excluding portions at the amino-terminus, carboxy-terminus or between the amino-terminus and carboxy-terminus of the complete protein. Examples of such fragments includes amino terminal fragmentation to eliminate the first five amino acids VPIHK of the rat *ob* gene used to create an *ob* monomer fragment or *ob* monomer fusion fragment and corresponding *ob* dimers or

15 *ob* dimer fusion proteins, amino terminal fragmentation to eliminate the first five amino acids VPIQK of the human *ob* gene used to create an *ob* monomer fragment or *ob* monomer fusion fragment and corresponding *ob* dimers or *ob* dimer fusion proteins, and amino terminal fragmentation to eliminate the first five amino acids VPIQK of the mouse *ob* gene used to create an *ob* monomer fragment or *ob*

20 monomer fusion fragment and corresponding *ob* dimers or *ob* dimer fusion proteins. Other examples of such fragments includes amino terminal fragmentation to eliminate the first twenty-nine amino acids of the rat *ob* gene used to create an *ob* monomer fragment or *ob* monomer fusion fragment starting with the sequence VSARQ and corresponding *ob* dimers or *ob* dimer fusion proteins, amino terminal

fragmentation to eliminate the first twenty-nine amino acids of the human *ob* gene used to create an *ob* monomer fragment or *ob* monomer fusion fragment starting with the sequence VSSKQ and corresponding *ob* dimers or *ob* dimer fusion proteins, and amino terminal fragmentation to eliminate the first twenty-nine amino acids of  
5 the mouse *ob* gene used to create an *ob* monomer fragment or *ob* monomer fusion fragment starting with the sequence VSAKQ and corresponding *ob* dimers or *ob* dimer fusion proteins. Variations in the products of the invention also include insertion of non-peptide bonds in the peptide backbone known in the art not to influence biological activity.

10 The invention also includes other modified *ob* dimers which conserve relevant activity, for example the anti-feeding activity, of the *ob* dimer. These are typically recombinant proteins that include hybrid proteins, such as fusion proteins, proteins resulting from the expression of multiple genes within the expression vector, proteins resulting from expression of multiple genes within the chromosome  
15 of the host cell, and may include a protein having relevant activity of a disclosed protein, for example anti-feeding activity, linked by peptide bonds to a second protein or peptide. For example, the present invention provides for the manufacture and use of dimeric and monomeric forms of the *ob* gene product that are active in vivo notwithstanding that they have fused to them certain marker peptides (such as  
20 poly-histidine, or the eight amino acid FLAG peptide described below) that are useful in the isolation and purification of both the dimeric and monomeric forms of a recombinant *ob* gene product. In addition to these fusion proteins which include marker or reporter peptides of various lengths, other fusion proteins of the invention include an *ob* protein having an amino acid sequence added to the N-terminus, for

example, to the N-terminus of a mouse, rat or human *ob* protein or protein fragment. This sequence may be linked to the *ob* protein by a peptide bond, a peptide bond surrogate or an appropriate chemical linker. More specifically, a sequence of amino acids between 1 and 200 residues long, preferably between 5 and 50 residues long, 5 having a balance of hydrophilicity and hydrophobicity such that the overall solution and other physicochemical properties of the fusion protein remain roughly within the range defined by the physicochemical properties of vertebrate *ob* proteins and protein fragments, such as mouse, rat and human *ob* proteins and protein fragments. Such sequences would result in a range for the pI value of the resulting fusion 10 protein of between 4 and 8. N-terminal and C-terminal fusion sequences include, but are not limited to, the following: amylin, including amylin analogues (for example, [Pro<sup>25,28,29</sup>]human amylin); calcitonins, including salmon calcitonin; cholecystokinins, including CCK-8; ceruletide; bombesin; enterogastrone; somatostatin; thyrotropin releasing hormone; segments of neuropeptide-Y which 15 possess neuropeptide-Y antagonist properties; neurotensin;  $\alpha$ -MSH;  $\beta$ -endorphins; glucagon; GLP-1; insulin; insulin like growth factor; TNFs; interleukins; apolipoprotein A-IV; and, peptide sequences which are known to target particular cells or tissues (R. Pasqualini and E. Ruoslahti, *Nature*, 380:364 (1996)). Fusion *ob* monomers and dimers and fragments thereof which include an N-terminal amylin, 20 an amylin analogue, a cholecystokinin, or a calcitonin are presently preferred.

The *ob* dimers and *ob* dimer fusion proteins of the present invention, as well as their corresponding *ob* monomers and *ob* fusion monomer proteins, also include variants of the *ob* domain amino acid sequence or sequences that differ only by conservative amino acid substitution and conserve the anti-feeding activity of the



isolated *ob* dimers or *ob* dimer fusion proteins. Conservative amino acid substitutions are defined as "sets" in Table 1 of Taylor, W.R., (1986) *J. Mol. Biol.*, 188: 233.

It will be appreciated by those in the art that there are various methods useful  
5 in preparing and isolating *ob* dimers and *ob* dimer fusion proteins (as well as their corresponding *ob* monomer and *ob* monomer fusion proteins). In general, recombinant DNA isolation techniques are now well known. An extensive discussion embodying a number of commonly used methodologies can be found in Sambrook et al., *Molecular Cloning, A Laboratory Manual, Second Edition*,  
10 Volumes 1 to 3, Cold Spring Harbor Laboratory Press 1989). Recombinant methods allow segments of genetic information, DNA, from different organisms, to be joined together outside of the organisms from which the DNA was obtained and this hybrid DNA to be incorporated into a cell that will allow the production of the protein for which the original DNA encodes. Genetic information encoding a protein of the  
15 present invention may be obtained from genomic DNA, mRNA (preferably adipose tissue mRNA), or tissue total RNA (preferably adipose tissue total RNA) of an organism by methods well known in the art. Preferred methods of obtaining this genetic information include isolating mRNA from an organism, converting it to its complementary DNA, incorporating the cDNA into an appropriate cloning vector,  
20 and identifying the clone which contains the cDNA encoding the desired protein by means of hybridization with appropriate oligonucleotide probes constructed from known or postulated sequences of the protein. Especially preferred methods of obtaining this genetic information include isolating tissue total RNA, preferably adipose tissue total RNA, from an organism, converting it to its complementary

DNA, and amplifying, detecting and isolating a cDNA sequence encoding the desired protein. The genetic information in the cDNA encoding a protein of the present invention may be ligated into an expression vector, the vector introduced into host cells, and the genetic information expressed as the protein encoded for.

5           Thus, nucleic acid encoding the proteins of the invention may be cloned by incorporating a DNA fragment coding for an *ob* protein or *ob* fusion protein in a recombinant DNA vehicle, typically, for example, mammalian, bacterial or viral vectors, and transforming a suitable host, for example, an *E. coli* cell line and isolating clones incorporating the recombinant vectors. Such clones may be grown  
10 and used to produce the desired *ob* dimer or *ob* dimer fusion protein or *ob* monomer fusion protein.

Mixtures of mRNA can be isolated from eukaryotic cells and double-stranded DNA copies of entire genes synthesized which are complementary to the isolated mRNA. mRNA is first reverse-transcribed to form a single-stranded cDNA  
15 by an RNA-directed DNA polymerase, *e.g.*, reverse transcriptase. Reverse transcriptase synthesizes DNA in the 5' to 3' direction, utilizes deoxyribonucleoside 5'-triphosphates as precursors, and requires both a template and a primer strand. By a series of additional reactions, double-stranded cDNA is produced and inserted into cloning or expression vectors by any one of many known techniques, which depend  
20 at least in part on the vector selected. Expression vectors refer to vectors which are capable of transcribing and translating DNA sequences contained therein, where such sequences are linked to other regulatory sequences capable of effecting their expression. These expression vectors are replicable in the host organisms or systems as either plasmids, bacteriophage, or as an integral part of the chromosomal DNA.

Recombinant vectors and methodology are in general well known and suitable for use in host cells over a wide range of prokaryotic and eukaryotic organisms. The cDNA cloning and expression procedures further described below and in the Examples are but some of a wide variety of well established methods to produce  
5 specific sequences and reagents useful in the invention.

One method of preparing the products of the invention includes the steps of constructing a vertebrate cDNA library, preferably a vertebrate adipose cDNA library; ligating the cDNA library into a cloning vector; introducing the cloning vector containing the cDNA library into a first host cell; contacting the cDNA  
10 molecules of the first host cell with a solution containing a suitable *ob* gene hybridization probe; detecting and then isolating a cDNA molecule which hybridizes to the *ob* gene hybridization probe and encodes the desired *ob* protein; ligating the hybridizing cDNA molecule into an expression vector; transforming a second host cell with the expression vector containing the cDNA molecule which encodes the  
15 desired *ob* protein; culturing the transformed second host cell under conditions that favor the production of the *ob* protein as a dimer; and, isolating the *ob* protein expressed by the second host cell.

The products of the invention may also be prepared by methods that do not require the construction and screening of a cDNA library. Such a method which  
20 represents one embodiment for the production of a rat *ob* fusion dimer is described in Examples 1, 2 and 4-7. Examples 1 and 2 describe methods used to isolate and clone the rat *ob* gene from rat adipose tissue total RNA using an RT-PCR amplification technique. Examples 4-7, 9-19, 22, 24 and 25 describe methods useful for the expression and isolation of the *ob* gene products in *E. coli*. The *ob* dimers,

*ob* dimer fusion proteins and *ob* monomer fusion proteins of the invention can be isolated by various methods or combinations of methods of protein purification as disclosed below. These purification methods are also useful for the isolation of unfused *ob* monomer proteins.

5 Preferred natural sources of mRNA from which to construct a cDNA library are vertebrate adipose tissue, for example, epididymal adipose tissue. Preferred methods of isolating mRNA encoding a protein of the present invention, along with other mRNA, from an mRNA source include poly U or poly dT chromatography. Other methods for RNA extraction, including the acid guanidinium thiocyanate  
10 procedure used in Example 1, which details the extraction of rat adipose tissue total RNA and the preparation of oligonucleotide primers for use in the isolation and cloning of the rat *ob* gene, are known in the art.

Preferred methods of obtaining double-stranded cDNA from isolated mRNA include synthesizing a single-stranded cDNA on the mRNA template using a reverse  
15 transcriptase, degrading the RNA hybridized to the cDNA strand using a ribonuclease (RNase), and synthesizing a complementary DNA strand by using a DNA polymerase to give a double-stranded cDNA. Especially preferred methods of preparing cDNA include those described in Example 2 wherein total RNA isolated from vertebrate adipose tissue is converted into single-stranded cDNA using Murine  
20 Leukemia Virus Reverse Transcriptase and RNase inhibitor, followed by a PCR procedure to amplify the target cDNA, yielding double-stranded cDNA.

cDNA encoding a protein of the present invention, along with the other cDNA if a library is constructed as above, are then ligated into cloning vectors. Cloning vectors include a DNA sequence which accommodates the cDNA. The

vectors containing the amplified cDNA or cDNA library are introduced into host cells that can exist in a stable manner and provide an environment in which the cloning vector is replicated. Suitable cloning vectors include plasmids, bacteriophages, viruses and cosmids. Preferred cloning vectors include plasmids.

5 Cloning vectors which are especially preferred in the isolation methods described herein for the preparation of RT-PCR products from total adipose tissue RNA include the plasmid pAMP 1.

The construction of suitable cloning vectors containing cDNA and control sequences employs standard ligation and restriction techniques which are well

10 known in the art. Isolated plasmids, DNA sequences or synthesized oligonucleotides are cleaved, tailored and religated in the form desired. With respect to restriction techniques, site-specific cleavage of cDNA is performed by treating with suitable restriction enzyme under conditions which are generally understood in the art, and particulars of which are specified by the manufacturers of these

15 commercially available restriction enzymes. *See, e.g.*, the product catalogs of New England Biolabs, Promega, and Stratagene Cloning Systems.

Cloning vectors containing the desired cDNA are introduced into host cells and cultured. Cloning vectors containing a cDNA library prepared as disclosed are introduced into host cells, the host cells are cultured, plated, and then probed with a

20 hybridization probe to identify clones which contain the recombinant cDNA encoding a protein of the present invention. Preferred host cells include bacteria when plasmid cloning vectors are used. Especially preferred host cells include *E. coli* strains such as *E. coli* DH5 $\alpha$ MCR competent cells.

Hybridization probes and primers are oligonucleotide sequences which are complementary to all or part of the cDNA molecule that is desired. They may be prepared using any suitable method, for example, the phosphotriester and phosphodiester methods, described respectively in Narang *et al.*, *Methods in Enzymology*, 68: 90 (1979) and Brown *et al.*, *Methods in Enzymology*, 68: 109 (1979), or automated embodiments thereof. In one such embodiment, diethylphosphoramidites are used as starting materials and may be synthesized as described by Beaucage *et al.*, *Tetrahedron Letters*, 22: 1859-1862 (1981). One method for synthesizing oligonucleotides on a modified solid support is described in U.S. Patent No. 4,458,066. Probes differ from primers in that they are labeled with an enzyme, such as horseradish peroxidase, or with a radioactive atom, such as  $^{32}\text{P}$ , to facilitate their detection. A synthesized probe is radio-labeled by nick translation using *E. coli* DNA polymerase I or by end labeling using alkaline phosphatase and T4 bacteriophage polynucleotide kinase.

Useful hybridization probes and amplification primers include oligonucleotide sequences which are complementary to a stretch of the cDNA encoding a portion of the amino acid sequence of an *ob* protein, for example, a portion of the amino acid sequence shown in Figure 1. Especially preferred as hybridization probes are oligonucleotide sequences encoding substantially all of the amino acid sequence of rat, mouse, or human *ob* protein. Other appropriate probes for isolation of vertebrate *ob* genes will be apparent to those skilled in the art. Especially preferred as amplification primers are pairs of oligonucleotide sequences that flank substantially all of the DNA sequence encoding a vertebrate *ob* protein, for example, those encoding rat, mouse, or human *ob* protein. A preferred cDNA

molecule encoding a vertebrate protein of the present invention can be identified by screening or amplification methods through its ability to hybridize to these probes or primers.

Upon identification of the clone containing the desired cDNA, whether by an  
5 RT-PCR procedure or through cDNA library screening, for example, amplification may be used to produce large quantities of a gene encoding a protein of the present invention in the form of a recombinant cDNA molecule. Preferred methods of amplification include the use of the polymerase chain reaction (PCR). *See, e.g., PCR Technology*, W.H. Freeman and Company, New York (Edit. Erlich, H.A.  
10 1992). PCR is an in vitro amplification method for the synthesis of specific DNA sequences. In PCR, two oligonucleotide primers that hybridize to opposite strands and flank the region of interest in the cDNA of the clone are used. A repetitive series of cycles involving cDNA denaturation into single strands, primer annealing to the single-stranded cDNA, and the extension of the annealed primers by DNA  
15 polymerase results in numbers of copies of cDNA, whose termini are defined by the 5' ends of the primers, approximately doubling at every cycle. Through PCR amplification, the coding domain and any additional primer encoded information such as restriction sites or translational signals (signal sequences, start and/or stop codons) of the recombinant cDNA molecule to be isolated is obtained. Preferred  
20 conditions for amplification of cDNA are found in manufacturer protocols, and may be accomplished manually or by automated thermocycling devices. An example of a cDNA prepared in this fashion is that having the nucleic acid sequence of Figure 1.

The cDNA molecules of the present invention when isolated as described are used to obtain expression of the *ob* dimers and *ob* dimer fusion proteins described

and claimed herein, as well as their corresponding *ob* fusion monomer proteins. Generally, a recombinant cDNA molecule of the present invention is incorporated into an expression vector, this expression vector is introduced into an appropriate host cell, the host cell is cultured, and the expressed protein is isolated. Various  
5 methods for *ob* dimer, *ob* monomer fusion protein and *ob* dimer fusion protein expression are described in Examples 3, 4, 9, 10, 12, 13, 15, 16, and 22.

Expression vectors are DNA sequences that are required for the transcription of cloned copies of genes and translation of their mRNAs in an appropriate host. These vectors can express either procaryotic or eucaryotic genes in a variety of cells  
10 such as bacteria, yeast, mammalian, plant and insect cells. Proteins may also be expressed in a number of virus systems.

Suitably constructed expression vectors contain an origin of replication for autonomous replication in host cells, or are capable of integrating into the host cell chromosomes. Such vectors will also contain selective markers, a limited number of  
15 useful restriction enzyme sites, a high copy number, and strong promoters. Promoters are DNA sequences that direct RNA polymerase to bind to DNA and initiate RNA synthesis; strong promoters cause such initiation at high frequency. The preferred expression vectors of the present invention are operatively linked to a cDNA or recombinant cDNA of the present invention, *i.e.*, the vectors are capable of  
20 directing both replication of the attached cDNA or recombinant cDNA molecule and expression of the protein encoded by the cDNA or recombinant cDNA molecule. Expression vectors may include, but are not limited to cloning vectors, modified cloning vectors and specifically designed plasmids or viruses. With each type of host cell certain expression vectors are preferred, as described below.



Procaryotes may be used and are presently preferred for expression of the *ob* dimers, *ob* monomer fusion proteins and *ob* dimer fusion proteins of the present invention. Suitable bacteria host cells include the various strains of *E. coli*, *Bacillus subtilis*, and various species of *Pseudomonas*. In these systems, plasmid vectors  
5 which contain replication sites and control sequences derived from species compatible with the host are used. Suitable vectors for *E. coli* are derivatives of pBR322, a plasmid derived from and *E. coli* species by Bolivar et al., *Gene*, 2: 95 (1977). Common procaryotic control sequences, which are defined herein to include promoters for transcription, initiation, optionally with an operator, along with  
10 ribosome binding site sequences, include the beta-lactamase and lactose promoter (Chang et al., *Nature*, 198: 1056 (1977)), the tryptophan promoter system (Goeddel et al., *Nucleic Acids Res.*, 8: 4057 (1980)) and the lambda-derived P<sub>L</sub> promoter and N-gene ribosome binding site (Shimatake et al., *Nature*, 292: 128 (1981)). However, any available promoter system compatible with procaryotes can be used.  
15 Preferred procaryote expression systems include *E. coli* and their expression vectors, such as *E. coli* strains W3110 and JM105, with suitable vectors, as described in Example 5. Especially preferred is the use of *E. coli* strain BL21(DE3), with suitable vectors, as described in Examples 9, 12, 15, 22 and 24.

Eucaryotes may be used for expression of the proteins of the present  
20 invention. Eucaryotes are usually represented by the yeast and mammalian cells. Suitable yeast host cells include *Saccharomyces cerevisiae* and *Pichia pastoris*. Suitable mammalian host cells include COS and CHO (Chinese Hamster Ovary) cells. Expression vectors for the eucaryotes are comprised of promoters derived from appropriate eucaryotic genes. Suitable promoters for yeast cell expression

vectors include promoters for synthesis of glycolytic enzymes, including those for the 3-phosphoglycerate kinase gene in *Saccharomyces cerevisiae* (Hitzman et al., *J. Biol. Chem.*, 255: 2073 (1980)) and those for the metabolism of methanol such as the alcohol oxidase gene in *Pichia pastoris* (Stroman et al., U.S. Patent Nos. 5 4,808,537 and 4,855,231). Other suitable promoters include those from the enolase gene (Holland et al., *J. Biol. Chem.*, 256: 1385 (1981)) or the Leu2 gene obtained from YEp13 (Broach et al., *Gene*, 8: 121 (1978)).

Suitable promoters for mammalian cell expression vectors include the early and late promoters from SV40 (Fiers et al., *Nature*, 273: 113 (1978)) or other viral 10 promoters such as those derived from polyoma, adenovirus II, bovine papilloma virus or avian sarcoma viruses. Suitable viral and mammalian enhancers may also be incorporated into these expression vectors.

Suitable promoters for plant cell expression vectors include the nopaline synthesis promoter described by Depicker et al., *Mol. Appl. Gen.*, 1: 561 (1978). 15 Suitable promoters for insect cell expression vectors include modified versions of the system described by Smith et al., U.S. Patent No. 4,475,051. The expression vector comprises a baculovirus polyhedrin promoter under whose control a cDNA molecule encoding a protein can be placed.

Another method of producing an *ob* dimer comprises the steps of culturing a 20 transformed host cell containing a DNA sequence encoding a vertebrate *ob* protein, preferably a human *ob* protein, under conditions that favor the production of said vertebrate *ob* protein as a dimer, and isolating the *ob* dimer expressed by the transformed host cell. Such a method is exemplified for the rat *ob* protein in Examples 5-7. Still another method of producing a recombinant *ob* dimer comprises

the steps of culturing a transformed host cell containing a DNA sequence encoding a vertebrate *ob* protein, preferably a human *ob* protein, under conditions that favor the production of said vertebrate *ob* protein as a monomer, isolating the *ob* protein expressed by the transformed host cell, and dimerizing the *ob* protein.

- 5           Dimerization may be achieved by initially treating *ob* monomer protein with a reducing agent such as mercaptoethanol or dithiothreitol in an appropriate buffer at pH 6-9. It is assumed that some refolding is required prior to dimerization and so the whole is diluted by 5-10 fold, or dialyzed with an appropriate buffer at pH 6-9, and allowed to equilibrate at 4°C. Ammonium bicarbonate buffer is preferred.
- 10          Incubation and air oxidation then yields dimeric protein. Other methods of oxidation typically used are O<sub>2</sub>/copper, mercury salts, etc. Folding aids can also be used, including other proteins such as albumins, chaperones, monoclonal antibodies, and soluble receptors, along with a variety of chromatography supports and plastic surfaces. Data also indicate, by way of example, that monomeric FLAG-*ob* protein
- 15          can be converted to dimeric FLAG-*ob* protein by incubation in Tris buffer at pH 7-9.

- Ob* dimer fusion proteins may be produced using similar methods. One such method for the recombinant production of an *ob* dimer fusion protein comprises the steps of constructing a vertebrate cDNA library, preferably a vertebrate adipose cDNA library, and more preferably a human adipose cDNA library; ligating the
- 20          cDNA library into a cloning vector; introducing the cloning vector containing the cDNA library into a first host cell; contacting the cDNA molecules of the first host cell with a solution containing a suitable *ob* gene hybridization probe; detecting a recombinant cDNA molecule which hybridizes to the probe; isolating the recombinant cDNA molecule; ligating the nucleic acid sequence of the cDNA

molecule which encodes an *ob* protein to a marker DNA sequence to create a fusion DNA sequence; ligating the fusion DNA sequence into an expression vector; transforming a second host cell with the expression vector containing the fusion DNA sequence; culturing the transformed second host cell under conditions that  
5 favor the production of the *ob* fusion protein as a dimer; and, isolating the *ob* protein expressed by the second host cell. As noted, the use of adipose tissue total RNA, followed by conversion to cDNA and amplification of the desired *ob* gene sequence may be used rather than steps involving the preparation and screening of a cDNA library or libraries, and is presently preferred.

10 A preferred peptide for preparation of an *ob* dimer fusion peptide is the FLAG peptide. See Hopp et al., A Short Polypeptide Marker Sequence Useful for Recombinant Protein Identification and Purification, (1988) *Biotechnology*, 6: 1205-1210. FLAG is an octapeptide with the amino acid sequence DYKDDDDK. Antibodies are available which specifically recognize this sequence, thus allowing  
15 identification (by Western Blotting) and purification (by affinity chromatography) of proteins containing this sequence. See Prickett et al., A Calcium Dependent Antibody for Identification and Purification of Recombinant Proteins, (1989) *BioTechniques*, 7: 580-589. In addition, the sequence may be specifically removed with enterokinase if placed at the N-terminus of the desired peptide. Thus, this  
20 particular reporter peptide allows identification, purification and liberation of a given protein to which it is attached. Additionally, cloning into the pFLAG-ATS vector (Scientific Imaging Systems, Eastman Kodak Company) allows periplasmic expression of an N-terminally tagged FLAG fusion protein in bacteria.

Another method of producing an *ob* dimer fusion protein comprises the steps of culturing a transformed host cell containing a DNA sequence encoding a vertebrate *ob* protein, preferably a human *ob* protein, coupled to a marker or other fusion DNA sequence under conditions that favor the production of said vertebrate  
5 *ob* fusion protein as a dimer, and isolating the *ob* dimer fusion protein expressed by the transformed host cell. Still another method of producing an *ob* dimer fusion protein comprises the steps of culturing a transformed host cell containing a DNA sequence encoding a vertebrate *ob* protein, preferably a human *ob* protein, coupled to a marker DNA sequence under conditions that favor the production of said  
10 vertebrate *ob* fusion protein as a monomer, isolating the *ob* fusion protein expressed by the transformed host cell, and dimerizing the *ob* fusion protein, as described above.

Intracellular and periplasmic expression using *E. coli* are preferred for *ob* protein expression. A number of recombinant production methods are described by  
15 contributors to Protein Purification - Micro to Macro, R. Burgess ed., Alan R. Liss, Inc., New York, 1987, and examples of periplasmic expression of recombinant proteins are given by H. Lee and P. Troota in Purification and Analysis of Recombinant Proteins, R. Seetharam and S. Sharma ed. Marcel Dekker, Inc., New York, 1991, p. 163-181. Provided herein are preferred methods for the periplasmic  
20 expression and purification of *ob* dimer protein, *ob* dimer fusion protein, *ob* monomer protein, and *ob* fusion monomer protein, which provide increased protein yield and quality. These methods include the use of a T7 promoter vector construct transfected into *E. coli* BL21(DE3) cells which are grown at about 25° to about 30°C in media containing a supplemental carbon source, preferably glucose, for enhanced

expression. Preferred purification methods include the use of an osmotic shock protocol which incorporates one or more specific protease inhibitors, preferably Peflabloc SC, followed by the addition of BisTris-propane, or buffers of a similar nature, and separation using a cellulose-based anion exchange chromatography resin, preferably DE-52 resin. Further purification may be undertaken using high pressure liquid chromatography, preferably reversed phase high pressure liquid chromatography. Such methods are described in the below Examples.

Intracellular expression can be used to make proteins in *E. coli*, but the production process is complicated by the need to dissolve the inclusion bodies using chaotropic agents and the difficulties inherent in refolding disulfide-bonded proteins, as discussed in Protein Refolding, G. Georgiou and E. Bernardez-Clark eds., (1991), American Chemical Society, Washington, DC. Also provided herein are preferred methods for the intracellular expression (into inclusion bodies) of recombinant *ob* proteins, and their subsequent solubilization, refolding and purification, which provide greatly increased protein yield in *E. coli*. In this method, certain naturally-occurring nucleotides within the codons for Val<sup>22</sup> and Pro<sup>23</sup> in the coding sequences for any of the mammalian *ob* proteins, including human, rat and mouse (which are not part of the set of deleterious codons AGG/AGA, CUA, AUA, CGA, or CCC, described by J. Kane, Curr. Opin. Biotechnol. (1995), 6:494-500), are replaced. An improved human DNA sequence coding for an *ob* protein which contains an additional nucleotide change in the codon for Gln<sup>23</sup> has also been invented and is described herein. These changes resulted in improved protein expression compared to a construct without these codon modifications, as described in Examples 16 and 25. Solubilization, refolding and purification of the

recombinant proteins are accomplished by lysing the cells and washing inclusion bodies in an anionic buffer of approximately neutral pH, preferably 100mM phosphate at a pH of about 6.5, dissolving the inclusion bodies in a buffer containing a chaotropic agent, such as urea in ammonium bicarbonate buffer, transferring the  
5 protein by dialysis or dilution into BisTris-propane or a similar buffer, and purifying the protein using a cellulose-based anion exchange chromatography resin, preferably DE-52 resin. The invention also provides for the preparation of *ob* monomer protein or *ob* monomer fusion protein without unwanted dimer formation by the addition of agents such as glutathione and dithiothreitol to any or all of the above-noted buffers.  
10 Further purification may be undertaken using high pressure liquid chromatography, preferably reversed phase high pressure liquid chromatography.

While recombinant DNA methods of production are preferred, chemical synthesis, using a solid phase synthesis approach or a combination of both solid phase peptide synthesis and solution chemistries offers a further method of  
15 preparation of *ob* products of the invention, including *ob* dimers. Examples of solid phase peptide synthesis include that described by Merrifield, *J. Amer. Chem. Soc.*, 85: 2149 (1964), or other equivalent methods known in the chemical arts, such as the method described by Houghten, *Proc. Natl. Acad. Sci.*, 82: 5132 (1985).

Solid phase synthesis is commenced from the C-terminus of the peptide by  
20 coupling a protected amino acid or peptide to a suitable insoluble resin. Suitable resins include those containing chloromethyl, bromomethyl, hydroxymethyl, aminomethyl, benzhydryl, and t-alkyloxycarbonylhydrazide groups to which the amino acid can be directly coupled. In one embodiment of this solid phase synthesis, for example, the carboxy terminal amino acid, having its alpha amino

group and, if necessary, its reactive side chain group suitably protected, is first coupled to the insoluble resin. After removal of the alpha amino protecting group, such as by treatment with trifluoroacetic acid in a suitable solvent (BOC chemistry) or piperidine (Fmoc chemistry), the next amino acid or peptide, also having its  
5 alpha amino group and, if necessary, any reactive side chain group or groups suitably protected, is coupled to the free alpha amino group of the amino acid coupled to the resin. Additional suitably protected amino acids or peptides are coupled in the same manner to the growing peptide chain until the desired amino acid sequence is achieved. The synthesis may be done manually, by using  
10 automated peptide synthesizers, or by a combination of these.

The coupling of the suitably protected amino acid or peptide to the free alpha amino group of the resin-bound amino acid can be carried out according to conventional coupling methods, such as the azide method, mixed anhydride method, DCC (dicyclohexylcarbodiimide) method, activated ester method (p-nitrophenyl  
15 ester or N-hydroxysuccinimide ester), BOP (benzotriazole-1-yl-oxy-tris (diamino) phosphonium hexafluorophosphate) method or Woodward reagent K method.

It is common in peptide synthesis that the protecting groups for the alpha amino group of the amino acids or peptides coupled to the growing peptide chain attached to the insoluble resin will be removed under conditions which do not  
20 remove the side chain protecting groups. Upon completion of the synthesis, it is also common that the peptide is removed from the insoluble resin, and during or after such removal, the side chain protecting groups are removed. Suitable protecting groups for the alpha amino groups of all amino acids, the omega amino group of lysine, the carboxy group of aspartic acid and glutamic acid, the guanidino



group of arginine, the thiol group of cysteine, the amide group of asparagine and glutamine, the imidazole group of histidine, the hydroxy group of serine and threonine, the indole group of tryptophan, and the phenyl hydroxy group of tyrosine are known in the art.

- 5           On use of the t-BOC method for protection/deprotection of the growing N-terminus, the completed peptide may be cleaved from the resin by treatment with liquid anhydrous hydrogen fluoride in ether containing one or more thio-containing carbocation scavengers at reduced temperatures. On use of the Fmoc method for protection/deprotection of the growing N-terminus, the completed peptide may be
- 10   cleaved from the resin by treatment with trifluoroacetic acid in water containing one or more carbocation scavengers at reduced temperatures. The cleavage of the peptide from the resin by such treatment in general will also remove all side chain protecting groups. The cleaved peptide is dissolved in water or acetonitrile/0.1%TFA/water and purified by conventional high pressure liquid
- 15   chromatography techniques, typically on a reverse phase column using trifluoroacetic acid-containing solvents. The purified peptide is then allowed to refold and establish proper disulfide bond formation by dilution to an appropriate peptide concentration, for example from about 0.025 mM to about 0.25 mM, in an appropriate buffer of pH 7-9 and then stirred open to air for about 24 to about 72
- 20   hours. In another manner, disulfide bond formation may be performed by using a protecting group such as Ac<sub>2</sub>m (acetamidomethyl) on the thiol group of a pair of cysteine residues and selectively cyclizing/deprotecting these protected amino acids with thallium trifluoroacetate in trifluoroacetic acid at reduced temperatures.

If necessary or desired, the refolded peptide is further purified by anion exchange chromatography carried out under essentially neutral conditions (pH 7-9), for example by Q-Sepharose anion exchange chromatography. Especially preferred is cellulose anion exchange chromatography, preferably employing a DE-52 resin.

- 5 Upon collection of fractions containing the purified peptide, the fractions are pooled and may be lyophilized to the solid peptide.

As indicated above, chemical synthesis may also be achieved by first preparing and then joining together peptide fragments. Typically, an initial choice is made as to which fragments offer the best possibility of providing a clean assembly  
10 of final product. See, e.g., Kent, *Angew. Chem. Int. Edit.*, 30, 113 (1991). Those skilled in the art will recognize the value, for example, of choosing to couple fragments with a glycine residue at the free C-terminus, which eliminates complications due to C-terminal racemization. The rat *ob* dimer may be prepared in this way. In the absence of signal sequence, the rat *ob* protein  
15 sequence has five glycine residues, all of which may be useful for fragment synthesis, leading to a six fragment approach. However, solid phase peptide synthesis allows the construction of relatively large fragments of up to 60 amino acids routinely. Thus, another choice of fragments includes but is not limited to the following three fragment approach:

20 Fragment 1

VPIHKVQDDT KTLIKTIVTR INDISHTQSV SARQRTGLD FIPG

Fragment 2

LHPILSLSKM DQTLAVYQQI LTLPSQNVL QIAHDLENLR DLLHLLAFSK  
SCSLPQTRG

25 Fragment 3

LQKPESLDGV LEASLYSTEV VALSRLO~~Q~~SL QDILQQLDLS PEC

Fully protected fragments as shown above are synthesized by standard solid phase peptide synthesis methods as described above on a polymeric resin support. Each are  
5 cleaved from the resin by standard methods which leave the protecting groups intact. Fragment 3 requires resin cleavage conditions which place a carboxylic acid protecting group at the C-terminus or requires subsequent protection at the C-terminus, whereas Fragments 1 and 2 require their N-terminal protecting groups remain intact. Fragment 3 also requires subsequent N-terminal deprotection while  
10 leaving side chain and C-terminal protecting groups intact.

The fragments are then assembled into full length protein as follows: The C-terminal carboxylic acid of N-terminal and side chain protected Fragment 2 is activated, typically with DCC (dicyclohexylcarbodiimide)/N-hydroxybenzotriazole in a suitable solvent, typically DMF (dimethylformamide). The activated Fragment 2  
15 is then reacted with C-terminal and side chain protected, N-terminal deprotected Fragment 3 for between 3 and 48 hours at room temperature. Alternatively, if water-DMF mixtures are used as solvents then a water soluble carbodiimide such as EDC (N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide) can be used in place of DCC. A standard workup gives the product which can be purified by reverse phase HPLC.  
20 Removal of the N-terminal protecting group of the assembled Fragment 2-3 is then required and can be achieved either before or after purification under conditions known to those skilled in the art.

The C-terminal carboxylic acid of N-terminal and side chain protected Fragment 1 is then activated, typically with DCC/N-hydroxybenzotriazole in a  
25 suitable solvent, typically DMF. The activated Fragment 1 is then reacted with C-

terminal and side chain protected, N-terminal deprotected Fragment 2-3 for between 3 and 48 hours at room temperature. Alternatively, if water/DMF mixtures are used as solvents then a water soluble carbodiimide such as EDC can be used in place of DCC. Again, a standard workup gives the product which can be purified by reverse  
5 phase HPLC.

Removal of all protecting groups of the assembled Fragment 1-2-3 is then required and is typically achieved by methods described above before. This is followed by purification, for example, by high pressure liquid chromatography or by anion exchange chromatography or cation exchange chromatography as described in  
10 Examples 7 and 14 below for the recombinant versions of the ob protein. Dimerization can then be achieved as described above or, if differential protection of the cysteines has been incorporated into the synthesis, by a sequence of selective deprotection, formation of one disulphide bond under standard conditions such as air oxidation or iodine oxidation in solution followed by further deprotection and  
15 disulphide bond formation. The choices made above are of course merely illustrative of, but not limited to, a strategy for chemical synthesis of the ob protein. Thus other permutations are envisaged, including a different order of coupling, the choice of other amino acid residues in the sequence as C-termini in fragments, and the choice of different fragments. The use of chemical ligation (Schnolzer and Kent, (1992)  
20 *Science*, 256: 221-225); Dawson *et al.*, (1994) *Science* , 266: 776-779), which involves fragment coupling under conditions such that a thio ester bond is inserted in the peptide backbone at chosen junctures is also envisaged.

The ob proteins of the present invention may be isolated, for example from host cell or media, by various methods known in the art, which include the use of

chromatographic methods, such as Q-Sepharose or Q-Sepharose HP anion chromatography, and suitable cation-exchange methods, such as SP-Sepharose chromatography, either alone or in sequential steps. See Examples 7 and 14. Preferred methods of purification of an *ob* monomer, *ob* dimer, *ob* monomer fusion  
5 protein and *ob* dimer fusion protein include chromatography of an extract through columns containing a cellulose-based anion-exchange resin, preferably DE-52, or containing a cellulose-based cation exchange resin, preferably CM-52. In the event that both anion and cation exchange chromatography methods are used it is preferred that the cation exchange chromatography be done before anion exchange  
10 chromatography. Presently preferred is the use of anion exchange chromatography with DE-52 resin for the initial purification of crude protein as described in Examples 11, 18, 22, 25 and 26.

Following an anion exchange chromatography procedure (or an anion exchange chromatography procedure preceded by cation exchange chromatography),  
15 a further purification using another chromatography method, for example, gel filtration chromatography using Sephacryl S100 or the like, hydrophobic interaction chromatography using Phenyl-Sepharose HP or the like, or reverse phase high pressure liquid chromatography, may be employed. Reverse phase high pressure liquid chromatography is preferred. See Example 19. The fractions collected after  
20 any such chromatography procedures may be selected by methods such as gel analysis, high pressure liquid chromatographic analysis, or by their ability to reduce feeding in mice, as described in Example 23 below, or by a combination of such analyses.

Another method of purification of *ob* monomer fusion protein or *ob* dimer fusion protein, such as a FLAG-*ob* monomer fusion protein or a FLAG-*ob* dimer fusion protein includes chromatography using an anti-FLAG affinity gel, provided by the manufacturer as a suspension of agarose beads covalently linked to anti-FLAG monoclonal antibodies, optionally followed by anion and/or cation exchange chromatography as described above, or by size fractionation using, for example, a Superose 12 gel, a Sephacryl S100 gel, or the like. In carrying out this affinity purification step, it is preferred to use CM-52 resin covalently linked to anti-FLAG monoclonal antibodies. The experiments of Example 6 indicated that capture of FLAG-*ob* protein on the FLAG M1 affinity resin could be limited by competition for binding sites between intact FLAG-*ob* protein and an N-terminal fragment of the FLAG-*ob* protein. Anion exchange fractionation may first be used to remove any FLAG containing fragments from the intact FLAG-*ob* monomer and dimer to improve the efficiency of the affinity purification step.

Especially preferred for the purification of *ob* proteins, including *ob* fusion monomer proteins, *ob* fusion dimer proteins, Met-*ob* proteins, and Met-*ob* fusion proteins, is cellulose resin-based anion exchange chromatography of the periplasmic extract, or the solubilized, refolded inclusion body protein, using for example, Whatman DE-52 resin, followed by a second chromatography step using reverse phase high pressure liquid chromatography. Preferred methods of purification of an *ob* dimer or *ob* dimer fusion protein, illustrated by purification of FLAG *ob* monomer and dimer fusion proteins, are provided in Examples 11.

Example 6 describes the purification of a FLAG-*ob* protein preparation containing a mixture of monomer and dimer, but predominantly monomer. Further

purification of monomer and dimer proteins to about 95% purity was accomplished by size exclusion chromatography using a Superose 12 gel size exclusion column.

Experiments described in Example 7 show methods useful for purification of *ob* monomers, *ob* dimers, *ob* monomer fusion proteins, and *ob* dimer fusion proteins.

- 5 These experiments detail the purification of FLAG-*ob* monomer and dimer proteins, demonstrating that anion exchange chromatography could be performed under essentially neutral (pH 7.4) conditions which closely resemble physiological conditions and were least likely to inactivate these proteins through unfolding or degradation. We also found anion exchange purification to be effective alone at
- 10 separating, for example, the monomeric and dimeric forms of the *ob*-FLAG protein. Additionally, we discovered that while elution of *ob* fusion protein from the FLAG affinity column with EDTA, pH 7.4, yields samples containing both *ob* monomer fusion protein and *ob* dimer fusion protein, but predominantly monomer, isolation and purification of *ob* dimer fusion protein to a purity of about 90% can be
- 15 accomplished by a first anion exchange chromatography step using Q-Sepharose or Q-Sepharose HP to obtain dimer fractions for FLAG affinity chromatography, followed by application of these fractions to a FLAG affinity column and employing EGTA at pH 8.2 as the elution buffer.

- The Example 7 results also showed that cation exchange chromatography
- 20 may give the largest purification gain over crude material, although the low pH needed for absorption of the FLAG-*ob* monomer and dimer proteins to the column material could result in unwanted unfolding or degradation.

The present invention also contemplates antibodies and immunoassays useful for detecting the presence or amount of an *ob* protein or protein fragment, for

example an *ob* dimer and/or *ob* dimer fusion protein, and antibodies useful therein. The general methodology and steps of antibody assays are described by Greene, U.S. Patent 4,376,110, entitled "Immunometric Assays Using Monoclonal Antibodies; Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory, Chapter 14

5 (1988); Radioimmunoassay and related methods", A. E. Bolton and W.M. Hunter, Chapter 26 of Handbook of Experimental Immunology, Volume I, Immunochemistry, edited by D.M. Weir, Blackwell Scientific Publications, 1986; "Enzyme immunoassays: heterogeneous and homogeneous systems", Nakamura, et al., Chapter 27 of Handbook of Experimental Immunology, Volume 1,

10 Immunochemistry, edited by D.M. Weir, Blackwell Scientific Publications, 1986; and Current Protocols in Immunology, Chapter 2, Section I, Edited by John E. Coligan, et al., (1991). In all such assays controls are preferably performed, which are designed to give positive and negative results. For example, the test may include a known *ob* dimer and/or *ob* dimer fusion protein positive control and a known non-

15 *ob* dimer and/or non-*ob* dimer fusion protein negative control. Other control samples may include an *ob* monomer or *ob* monomer fusion protein.

Preferably, the immunoassay is a sandwich immunoassay, and comprises the steps of (1) reacting an immobilized anti-*ob* dimer and/or anti-*ob* dimer fusion protein antibody, preferably a monoclonal antibody, and a labeled anti-*ob* dimer or

20 anti-*ob* dimer fusion protein antibody, preferably a monoclonal antibody, which recognizes a different site from that recognized by the immobilized antibody, with a sample containing or suspected of containing an *ob* dimer and/or *ob* dimer fusion protein so as to form a complex of immobilized antibody-*ob* dimer and/or *ob* dimer fusion protein-labeled antibody, and (2) detecting the presence or amount of *ob*



dimer and/or *ob* dimer fusion protein by determining the presence or amount of label in the complex. In this process the reaction of the immobilized antibody and labeled antibody with the sample may be carried out either simultaneously or separately. Alternatively, one of the antibody pair used in such an assay, preferably the labeled  
5 antibody, is an anti-*ob* monomer antibody or an anti-*ob* monomer fusion protein antibody. In assaying for *ob* dimer, it will be understood that both the labeled and bound antibody may be the same or different anti-*ob* monomer antibodies.

Suitable antibodies, for example anti-*ob* dimer and/or anti-*ob* dimer fusion protein antibodies, preferably monoclonal antibodies, in accordance with the present  
10 invention are specific to an *ob* dimer and/or *ob* dimer fusion protein over an *ob* monomer and/or *ob* monomer fusion protein. Such antibodies, as well as suitable anti-*ob* monomer antibodies or anti-*ob* monomer fusion protein antibodies, can be prepared from hybridomas by the following method. *Ob* dimers or *ob* dimer fusion proteins or fragments thereof, including those fragments described in Example 9, in  
15 an amount sufficient to promote formation of antibodies, are emulsified in an adjuvant such as Freund's complete adjuvant. The immunogen may be either crude or partially purified, and is administered to a mammal, such as mice, rats or rabbits, by intravenous, subcutaneous, intradermic, intramuscular, or intraperitoneal injection. In the preparation of polyclonal antibodies, after completion of the  
20 immunization protocol, sera are recovered from the immunized animals. In the preparation of monoclonal antibodies, after completion of the immunization protocol, as described for example in Example 8, animal spleens are harvested and myeloma cells having a suitable marker such as 8-azaguanine resistance can be used as parent cells which are then fused with the antibody-producing spleen cells to

prepare hybridomas. Suitable media for the preparation of hybridomas according to the present invention include media such as Eagle's MEM, Dulbecco's modified medium, and RPMI-1640. Myeloma parent cells and spleen cells can be suitably fused at a ratio of approximately 1:4. Polyethylene glycol (PEG) can be used as a suitable fusing agent, typically at a concentration of about 35% for efficient fusion. Resulting cells may be selected by the HAT method. Littlefield, J. W., (1964) *Science* 145: 709. Screening of obtained hybridomas can be performed by conventional methods including an immunoassay using culture supernatant of the hybridomas to identify a clone of hybridoma producing the objective immunoglobulin. The obtained antibody-producing hybridoma can then be cloned using known methods such as the limiting dilution method. In order to produce, for example, the anti-ob dimer and/or anti-ob dimer fusion protein monoclonal antibodies of the present invention, the hybridoma obtained above may be cultured either in vitro or in vivo. If the hybridoma is cultured in vitro, the hybridoma may be cultured in the above-mentioned media supplemented with fetal calf serum (FCS) for 3-5 days and monoclonal antibodies recovered from the culture supernatant. If the hybridoma is cultured in vivo, the hybridoma may be implanted in the abdominal cavity of a mammal, and after 1-3 weeks the ascites fluid collected to recover monoclonal antibodies therefrom. Much larger quantities of the monoclonal antibodies can efficiently be obtained using in vivo cultures rather than in vitro cultures and, thus, in vivo cultures are preferred. The monoclonal antibody obtained from the supernatant or ascites fluids can be purified by conventional methods such as ammonium sulfate-fractionation, Protein G-Sepharose column chromatography,

or their combinations. Illustrative and detailed methods for preparing the antibodies described and claimed herein are further provided in Example 8.

Antibodies, or the desired binding portions thereof including F(ab) and Fv fragments, along with antibody-based constructs such as single chain Fv's can also  
5 be generated using processes which involve cloning an immunoglobulin gene library in vivo. See, e.g., Huse et al., Generation of a Large Combinatorial Library of the Immunoglobulin Repertoire in Phage Lambda, (1989) *Science* 246: 1275-1281. Using these methods, a vector system is constructed following a PCR amplification of messenger RNA isolated from spleen cells with oligonucleotides that incorporate  
10 restriction sites into the ends of the amplified product. Separate heavy chain and light chain libraries are constructed and may be randomly combined to coexpress these molecules together and screened for antigen binding. Single chain antibodies and fragments may also be prepared by this method.

A sandwich immunoassay for *ob* dimers and/or *ob* dimer fusion proteins can  
15 suitably be prepared using an immobilized anti-*ob* dimer and/or anti-*ob* dimer fusion protein monoclonal antibody and a labeled anti-*ob* dimer and/or anti-*ob* dimer fusion protein monoclonal antibody. Anti-*ob* monomer antibodies and anti-*ob* monomer fusion protein antibodies can also be used as the immobilized or labeled antibody in conjunction with an anti-*ob* dimer and/or anti-*ob* dimer fusion protein monoclonal  
20 antibody to form the antibody/antigen/antibody sandwich. If an anti-*ob* monomer antibody or anti-*ob* monomer fusion protein antibody is used in the sandwich immunoassay to form a part of the antibody pair, it is preferably the labeled antibody. Antibodies according to the present invention can suitably be immobilized on commercially available carriers for the antigen-antibody reaction including beads,

balls, tubes, and plates made of glass or synthetic resin. Suitable synthetic resins include polystyrene and polyvinyl chloride. Anti-*ob* dimer, anti-*ob* monomer, anti-*ob* monomer fusion protein, and anti-*ob* dimer fusion protein monoclonal antibodies are suitably absorbed onto the carrier by allowing them to stand at 2-8°C overnight  
5 in 0.05M carbonate buffer, pH 9-10, preferably about pH 9.5. The immobilized anti-*ob* dimer, anti-*ob* monomer, anti-*ob* monomer fusion protein, and/or anti-*ob* dimer fusion protein monoclonal antibody can be stored cold in the presence of preservatives such as sodium azide. Both monoclonal and polyclonal antibodies can be immobilized onto carriers using this method.

10 Labeled anti-*ob* dimer, anti-*ob* monomer, anti-*ob* monomer fusion protein, and anti-*ob* dimer fusion protein antibodies in accordance with the present invention can suitably be prepared by labeling anti-*ob* dimer, anti-*ob* monomer, anti-*ob* monomer fusion protein, and anti-*ob* dimer fusion protein antibodies with any substance commonly used for an immunoassay including radioisotopes, enzymes,  
15 and fluorescent substrates. Radioisotopes and enzymes are preferably used. When radioisotopes are used as labels, the antibody is preferably labeled with <sup>125</sup>I using conventional methods such as the Chloramine T method (Hunter et al., *Nature* (1962) 194: 495) and the Bolton-Hunter method. When enzymes are used as labels, the antibody is labeled with an enzyme such as horseradish peroxidase, β-D-  
20 galactosidase, or alkaline phosphatase by conventional methods including the maleimide method and the Hingi method. Ishikawa et al., (1983) *J. Immunoassay* 4:  
1.

The activity of the label can be detected by conventional methods. If radioisotopes are used as labels, the activity of the label can be detected using an

appropriate instrument such as a scintillation counter. If enzymes are used as labels, the activity of the label can be detected by measuring absorbance, fluorescence intensity, or luminescence intensity after reacting the enzyme with an appropriate substrate.

5           The present invention also provides a kit for assaying the amount of *ob* dimer and/or *ob* dimer fusion protein present in a sample, including for example both biological samples and samples of *ob* dimers and/or *ob* dimer fusion proteins. The kit of the present invention comprises an immobilized anti-*ob* dimer and/or anti-*ob* dimer fusion protein monoclonal antibody and a labeled anti-*ob* dimer and/or anti-*ob* dimer fusion protein monoclonal antibody. When *ob* dimers and/or *ob* dimer fusion proteins are assayed using this kit, *ob* dimers and/or *ob* dimer fusion proteins become sandwiched between the immobilized monoclonal antibody and the labeled monoclonal antibody. As noted above, anti-*ob* monomer antibodies and anti-*ob* monomer fusion protein antibodies can also be used as the immobilized or labeled antibody to form one half of the antibody pair in conjunction with an anti-*ob* dimer and/or anti-*ob* dimer fusion protein monoclonal antibody.

          Since the monoclonal antibodies useful for this kit can optionally recognize *ob* dimers or *ob* dimer fusion proteins or both when they coexist, depending on the monoclonal antibodies used, the total amounts of *ob* dimers or *ob* dimer fusion proteins or both can be measured by this invention.

          The *ob* dimers, *ob* dimer fusion proteins, and *ob* monomer fusion proteins described and claimed herein have potent and prolonged food intake inhibition properties in vivo. These compounds will thus have significant utility in further

investigations seeking to elucidate the actions and physiological role of the *ob* gene in vivo.

Their properties also support the use of *ob* dimers, *ob* dimer fusion proteins, and *ob* monomer fusion proteins in the treatment of subjects with disorders or

5 conditions that would benefit from reduced food intake or increased energy expenditure. Such conditions or disorders include obesity and diabetes, particularly Type 2 diabetes. In particular, the compounds of the invention possess activity as anti-obesity agents, as evidenced by the ability to reduce feeding in mammals. As shown, for example, by the experiments in Example 23 below, [NEEDS TO BE

10 **REWRITTEN/UPDATED** -- the *ob* dimer is able to markedly depress food intake in fasted (and presumably hungry) mice for up to 12 hours following a single injection, with no apparent "catch-up" in food intake over 24 hours, and with no observable effects on behavior or well being, other than reduced food intake]. These unexpected and beneficial effects can be expected to provide a safe and effective

15 therapeutic control of eating and energy balance suitable for the chronic (long-term) amelioration of obesity by loss of excess adipose tissue, together with expected improvement in co-morbid conditions, including Type 2 diabetes. The *ob* dimers, *ob* dimer fusion proteins, and *ob* monomer fusion proteins thus may also be used to prepare pharmaceutical compositions, and used in methods for the treatment of

20 disorders and conditions which may benefit from the administration of such compositions. Such pharmaceutical compositions include therapeutically effective amounts of an *ob* dimer or *ob* dimer fusion protein or *ob* monomer fusion protein in pharmaceutically acceptable carriers. By "therapeutically effective amount" is meant an amount useful to cause reduced food intake or increased energy

expenditure or both. *Ob* dimers and *ob* dimer fusion proteins include human *ob* dimers and human *ob* dimer fusion proteins, rat *ob* dimers and rat *ob* dimer fusion proteins, mouse *ob* dimers and mouse *ob* dimer fusion proteins, as well as other vertebrate *ob* dimers and vertebrate *ob* dimer fusion proteins. *Ob* monomer fusion  
5 proteins include human *ob* monomer fusion proteins, rat *ob* monomer fusion proteins, mouse *ob* monomer fusion proteins, as well as other vertebrate *ob* monomer fusion proteins. Treatment methods comprise the administration of a therapeutically effective amount of a pharmaceutical composition comprising an *ob* dimer or *ob* dimer fusion protein or *ob* monomer fusion protein to patients in need  
10 thereof.

These pharmaceutical compositions may be administered separately or together with other compounds and compositions that may be useful in the treatment of said disorders and conditions, including but not limited to other compounds and compositions that comprise an amylin or an amylin agonist. See Lutz *et al.*, (1994)  
15 *Physiology and Behavior*, 55: 891-895. Suitable amylin agonists include, for example, human amylin and rat amylin. Suitable amylin agonists include, for example, <sup>25, 28</sup>  
<sup>29</sup>Pro-human amylin, amylin agonists as described in "Amylin Agonist Peptides and Uses Therefor," International Patent Application Number PCT/US92/09842 (published May 27, 1993), and salmon calcitonin.

20 The compounds referenced above, as well as *ob* monomer protein, form salts with various inorganic and organic acids and bases. Such salts include but are not limited to salts prepared with organic and inorganic acids; for example, HCl, HBr, H<sub>2</sub>SO<sub>4</sub>, H<sub>3</sub>PO<sub>4</sub>, trifluoroacetic acid, acetic acid, formic acid, methanesulfonic acid, toluenesulfonic acid, maleic acid, fumaric acid and camphorsulfonic acid. Salts

prepared with bases include ammonium salts, alkali metal salts, *e.g.*, sodium and potassium salts, and alkali earth salts, *e.g.*, calcium and magnesium salts. Acetate, hydrochloride, and ammonium bicarbonate salts are preferred. Ammonium bicarbonate salts are especially preferred, and are also preferred for preparation of *ob* monomer protein. The salts may be formed by conventional means, and by reacting the free acid or base forms of the product with one or more equivalents of the appropriate base or acid in a solvent or medium in which the salt is insoluble, or in a solvent such as water which is then removed in vacuo or by freeze-drying or by exchanging the ions of an existing salt for another ion on a suitable ion exchange resin, or gel filtration resin. See Examples 19, 20 and 22.

Compositions or products of the invention may conveniently be provided in the form of formulations suitable for parenteral (including intravenous, intramuscular and subcutaneous) or nasal or oral administration. In some cases, it will be convenient to provide an *ob* dimer or *ob* fusion protein or *ob* monomer fusion protein of the invention and another shorter-acting satiety agent, such as an amylin or an amylin agonist in a single composition or solution for administration together. Also contemplated is *ob* monomer protein an amylin or an amylin agonist in a single composition or solution for administration together. In other cases, it may be more advantageous to administer an amylin or an amylin agonist separately from the *ob* dimer or *ob* fusion protein or *ob* monomer fusion protein. A suitable administration format may best be determined by a medical practitioner for each patient individually. Suitable pharmaceutically acceptable carriers and their formulation are described in standard formulation treatises, *e.g.*, *Remington's Pharmaceutical Sciences* by E. W. Martin. See also Wang, Y. J. and Hanson, M. A.



"Parenteral Formulations of Proteins and Peptides: Stability and Stabilizers," *Journal of Parenteral Sciences and Technology*, Technical Report No. 10, Supp. 42:2S (1988). *Ob* dimers and *ob* fusion proteins should preferably be formulated in solution at neutral pH, for example about pH 6.5 to about pH 8.5, more preferably from about pH 7.5 to 8.5, with an excipient to bring the solution to about isotonicity, for example 4.5% mannitol or 0.9% sodium chloride, pH buffered with art-known buffer solutions, such as sodium phosphate, that are generally regarded as safe, together with an accepted preservative such as metacresol 0.1% to 0.75%, more preferably from about 0.1% to about 0.37% phenol. *Ob* dimers and *ob* dimer fusion proteins may not be stable or may be partially or wholly denatured at acid pH and thus pHs below 6 should be avoided as far as possible in manufacture, purification and formulation of these agents. The desired isotonicity may be accomplished using sodium chloride or other pharmaceutically acceptable agents such as dextrose, boric acid, sodium tartrate, propylene glycol, polyols (such as mannitol and sorbitol), or other inorganic or organic solutes. Sodium chloride is preferred particularly for buffers containing sodium ions.

Especially preferred lyophilized and liquid formulations for an *ob* dimer or *ob* fusion protein or *ob* monomer or *ob* monomer fusion protein are described in Examples 20 and 21. In a preferred lyophilized form, the proteins of the invention are prepared as ammonium bicarbonate salts. In a preferred liquid formulation, the proteins of the invention are prepared in BisTris-propane buffer, or buffers of similar structure, with or without a detergent, such as Tween 80, to have a pH from about 7.5 to about 9, most preferably a pH of 8.5. Lyophilized and liquid

formulations of *ob* monomer proteins may also be formulated as described herein and such formulations form a part of the invention.

A form of repository or "depot" slow release preparation may be used so that therapeutically effective amounts of the preparation are delivered into the  
5 bloodstream over many hours or days following transdermal injection or delivery.

If desired, solutions of the above compositions may be prepared in emulsified form, either water in oil or oil in water. Any of a wide variety of pharmaceutically acceptable emulsifying agents may be employed including, for example, acacia powder, a non-ionic surfactant (such as a Tween), or an ionic  
10 surfactant (such as alkali polyether alcohol sulfates or sulfonates, e.g., a Triton). If desired, solutions of the above compositions may also be prepared and dried in the presence of the sugar trehalose to enhance shelf life and stability. The therapeutically useful compositions of the invention are prepared by mixing the ingredients following generally accepted procedures. For example, the selected  
15 components may be mixed to produce a concentrated mixture which may then be adjusted to the final concentration and viscosity by the addition of water or thickening agent and possibly a buffer to control pH or an additional solute to control tonicity.

For use by the physician, the compositions will be provided in dosage unit  
20 form containing an amount of a compound of the invention which will be effective in one or multiple doses to reduce food intake and/or increase energy expenditure at a selected level. Therapeutically effective amounts of an *ob* dimer or *ob* dimer fusion protein or *ob* monomer or *ob* monomer fusion protein as described herein for the treatment of obesity, diabetes, and other such conditions in which food intake is

beneficially reduced and/or energy expenditure enhanced include those that decrease food intake, preferably to about fifty percent of pretreatment levels or such that food intake is reduced as desired. Compounds of the invention are preferably administered to provide peak plasma levels of *ob* dimer or *ob* dimer fusion protein or  
5 *ob* monomer or *ob* monomer fusion protein that are about twice the plasma levels of human *ob* protein observed in healthy subjects, and preferably that are about two to ten times the levels, who have been on a caloric-enriched diet, for example 50 to 80 kcal/kg for about one to four weeks. As will be recognized by those in the field, an effective amount of therapeutic agent will vary with many factors including the age  
10 and weight of the patient, the patient's physical condition, the *ob* protein level or decrease in food intake or increase in energy expenditure to be obtained, and other factors.

The effective dose of the *ob* dimer and *ob* dimer fusion protein compounds [MONOMER?] of this invention will typically be in the range of 0.02 mg to about  
15 2 mg once or twice per day, preferably about 0.05 mg to about 1 mg once per day on waking [ANY MODIFICATION TO THIS?]. As noted, the exact dose to be administered is determined by the attending clinician and is dependent upon where the particular compound lies within the above quoted range, as well as upon the age, weight and condition of the individual. A similar dosage range is provided for *ob*  
20 monomer fusion proteins [WILL THE FUSION MONOMER BE LESS? WHAT ABOUT THE FORMULATED UNFUSED MONOMER?].

The presently preferred mode of administration is by subcutaneous injection of a parenteral solution via a disposal syringe and needle, for example an insulin

syringe, or from a pre-filled cartridge fitted to a pen injector such as, by way of example, those provided by Becton-Dickinson for insulin injection.

To assist in understanding the present invention, the following Examples are provided which describe the results of a series of experiments. The experiments  
5 relating to this invention should not, of course, be construed as specifically limiting the invention and such variations of the invention, now known or later developed, which would be within the purview of one skilled in the art are considered to fall within the scope of the invention as described herein and hereinafter claimed.

10

### Examples

#### **Example 1 – Extraction of Rat RNA and Preparation of Oligonucleotide**

##### **Primers**

This Example describes the extraction of rat total RNA and the preparation of oligonucleotide primers for use in the isolation and cloning of the rat *ob* gene.

15 Epididymal adipose tissue was obtained from a male Sprague Dawley rat, immediately frozen in liquid nitrogen, and then stored at -80°C. Total RNA was extracted by the acid guanidinium thiocyanate procedure (Chomczynski, P. and Sacchi, N. (1987) *Analytical Biochemistry* 162: 156-159).

Oligonucleotides were synthesized for use as primers in PCR amplifications.  
20 Upstream primer A630 corresponds to positions 98-118 of mouse *ob* cDNA (Zhang, et al., (1994) *Nature* 372: 425-432) and was constructed with a non-gene-specific triplet repeat at its 5' end to facilitate subcloning. It has the following sequence: 5'-(CUA)<sub>4</sub>-AAGATCCCAGGGAGGAAAATG-3'. Downstream primer A631 corresponds to positions 637-617 of the non-coding strand of mouse *ob* cDNA (*id.*)

and also contains a non-gene-specific triplet repeat at its 5' end to facilitate subcloning. Its sequence is as follows: 5' (CAU)<sub>4</sub>-CTGGTGGCCTTTGAAACTTCA-3'. Oligonucleotides were synthesized on a MilliGen/Biosearch Cyclone Plus DNA synthesizer using standard  $\beta$ -cyanoethyl phosphoramidite chemistry. Following synthesis they were cleaved from the support, deprotected and eluted. After evaporation to dryness they were dissolved in water.

### Example 2 -- Rat *ob* cDNA Isolation and Cloning

This Example describes the methods used to isolate and clone rat cDNA coding for the rat *ob* protein using the primers and RNA of Example 1. The nucleotide and deduced amino acid sequences of the rat *ob* gene were then determined.

Rat *ob* cDNA was RT-PCR amplified from rat adipose tissue total RNA using reagents from the Perkin Elmer RNA PCR kit (Perkin Elmer, Foster City, CA). Conversion of RNA to cDNA was accomplished in a final volume of 100  $\mu$ l containing 10 mM Tris-HCl, pH 8.3 / 50 mM KCl / 5 mM MgCl<sub>2</sub> / 1 mM each dNTP / 5  $\mu$ g rat adipose tissue total RNA / 2.5  $\mu$ M random hexamers / 1 unit  $\mu$ l<sup>-1</sup> RNase inhibitor / 2.5 units  $\mu$ l<sup>-1</sup> Murine Leukemia Virus Reverse Transcriptase (MuLVRT). The reaction was heated for 5 min at 70°C and quick chilled on ice for 1 minute before the addition of the RNase inhibitor, random hexamers, and MuLVRT. The reaction was then covered with 75  $\mu$ l mineral oil and incubated as follows: 22°C for 10 min, 42°C for 1 hour, 99°C for 5 min, and then chilled on ice.

PCR amplification was accomplished using 20  $\mu$ l of the above cDNA synthesis reaction in a final 100  $\mu$ l volume containing 10 mM Tris-HCl, pH 8.3 / 50

mM KCl / 2.5 mM MgCl<sub>2</sub> / 0.2 mM each dNTP / 0.5 μM primer A630 / 0.5 μM primer A631 / 2.5 units AmpliTaq® DNA Polymerase. The reaction was incubated for 45 cycles of 1 min denaturation at 94°C, 1 min annealing at 48°C , and 1 min extension at 72°C, followed by a final incubation for 10 min at 72°C.

5        Products from the RT-PCR were resolved on a 2% agarose gel and a DNA fragment having a predicted 564 bp length was excised from the gel and purified using a silica membrane system (SpinBind® DNA Recovery System; FMC BioProducts, Rockland, ME) following the manufacturer's protocol. The purified DNA fragment was subcloned into plasmid vector pAMP 1 by a uracil DNA  
10       glycosylase methodology (CloneAmp™ System, GIBCO BRL, Gaithersburg, MD) following the manufacturer's protocol. The resulting products were used to transform *E. coli* DH5αMCR competent cells (GIBCO BRL) following the manufacturer's protocol. Plasmid DNA from transformants was purified using a minilysate procedure (Wizard™ Minipreps DNA Purification System, Promega,  
15       Madison, WI) and sequenced by a dideoxy chain termination method (ds Cycle Sequencing System, GIBCO BRL). The DNA sequence of rat *ob* clone pAMP1ROB#3 and its deduced amino acid sequence is presented in Figure 1. The triplet nucleotides for the initiation methionine and termination codons were  
20       introduced into the sequence by virtue of the primers employed in the PCR amplification.

### Example 3 – In vitro Transcription/Translation of the Rat *Ob* Gene

This Example describes the methods used in the in vitro transcription and translation of the rat *ob* gene isolated as set forth above.

One microgram of pAMPIROB#3 from Example 2 was used as a template for *in vitro* transcription/translation using the TnT Sp6 Coupled Rabbit Reticulocyte Lysate kit (Promega Corp.) following the manufacturer's protocol. A separate reaction was prepared containing canine pancreatic microsomal membranes (Promega Corp.) in the reaction mix. A third reaction was prepared without pAMPIROB#3 DNA template and served as a negative control. The translation products were labeled with L-(<sup>35</sup>S)methionine (Amersham Life Sciences Inc., # SJ.1015).

Aliquots of the reactions were run under reducing and non-reducing conditions on a 10-20% Tricine SDS polyacrylamide gradient gel (Novex Inc.). After electrophoresis, the gel was fixed in a solution of isopropanol/dH<sub>2</sub>O/acetic acid and then soaked in fluorography enhancer (Amplify; Amersham Life Sciences Inc.). The gel was subsequently dried and exposed to Kodak XAR film for 15 hours. A ~ 29,000 dalton band was observed from the reactions containing pAMPIROB#3 when run under non-reducing conditions. A second band, running slightly below the 29,000 dalton band, was observed in the reaction containing the microsomal membranes. These bands were not visible when the same samples were reduced prior to electrophoresis. Observation of bands running in the 16,000 to 18,000 dalton range was not possible due to non-specific background from the lysate.

In order to observe translation products in the 16,000 to 18,000 dalton range, the rat *ob* gene was subcloned, utilizing *Hind* III and *Sal* I restriction sites, from pAMPIROB#3 into vector pSP64poly(A) (Promega Corp.) creating pSP64ROBa. *In vitro* transcription/translation analysis was repeated, as outlined above, using the TnT Sp6 Coupled Wheat Germ Extract kit (Promega Corp.) and using the

manufacturer's protocol. A single band was observed running between the 16,525 and 18,800 dalton molecular weight markers, under both non-reducing and reducing conditions, specific to the reaction containing pSP64ROBa as template.

**Example 4 – *E. coli* Periplasmic Expression of the FLAG/Rat *ob* Gene**

5        The coding region for mature rat *ob* was PCR amplified from pAMP1ROB#3 using an upstream gene specific oligonucleotide (A638: 5'-AGTCCCTATCCACAAAGT  
CC-3') and a downstream oligonucleotide (A540) corresponding to the T7 promoter primer in the pAMP 1 vector. The first nucleotide of the upstream primer  
10 corresponds to the last nucleotide of the C-terminal Lysine codon of the FLAG epitope in vector pFLAG-ATS (IBI/Kodak Scientific Imaging Systems Inc.). The remainder of the upstream primer corresponds to the coding sequence of rat *ob* beginning with Valine-22. A single silent mutation (G to C) was incorporated into primer A638 at the third position of the Valine-22 codon in order to reconstruct the  
15 *Tth1111* I site in the multiple cloning site of the pFLAG-ATS vector.

PCR amplification was performed in a 100µl volume containing 30 ng pAMP1ROB#3, 0.5 µM each primer (A638, A540), 2.5 units of AmpliTaq DNA® Polymerase, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 0.2 mM each dNTP, and 2.5 mM MgCl<sub>2</sub>. The reaction was incubated for one cycle at 95°C/1 min, 51°C/1 min  
20 and 72°C/1 min followed by 30 cycles at 95°C/30 sec, 51°C/30 sec and 72°C/30 sec followed by a final incubation of 10 min at 72°C. Analytical agarose gel electrophoresis using 10 µl of the above 100 µl reaction mixture confirmed a single band of the expected size, 543 bp. The remaining PCR reaction material was purified using the QIAquick PCR Purification kit (QIAGEN Inc.) following the kit protocol.



The PCR fragment was digested with *EcoRI* and cloned into the pFLAG-ATS vector cut with *AspI* (an isoschizomer of *Tth111I*) and *EcoRI*. The resulting subclone, pFLAGROB#5, was sequenced using the N-26 primer (IBI/Kodak Scientific Imaging Systems) and various other primers specific to the rat *ob* sequence, to confirm proper construction of the coding region.

To test expression of the FLAG/rat *ob* fusion protein, pFLAGROB#5 in *E. coli* strain DH5 $\alpha$ F' (Gibco BRL) was grown at 37°C with rotation (250 rpm) in 20 mL of LB media containing 0.4% glucose and 50 $\mu$ g/mL ampicillin. When the OD<sub>600</sub> reached 0.75, IPTG was added to a final concentration of 0.5 mM and the incubation was continued for an additional 5 hours. One mL aliquots of the culture were collected at various time points throughout the incubation. The cells were pelleted, supernatant removed and the cells resuspended in 50  $\mu$ L of 2x Tricine SDS Loading Buffer, then heated for 5 min at 95°C. A duplicate cell pellet was resuspended in 400  $\mu$ L 0.5 M Sucrose/TE pH 8.0, pelleted in a centrifuge, and the supernatant was removed. Then the pellet was resuspended in 100  $\mu$ L ice-cold H<sub>2</sub>O followed by centrifugation at 4°C. The supernatant was collected to a new tube and an equal volume of 2x Tricine SDS Loading Buffer was added to the sample followed by incubation at 95°C for 5 minutes. This sample is indicative of the protein being transported into the periplasm of the *E. coli*. Aliquots of the cell samples were run under reducing and non-reducing conditions on a 10-20% Tricine SDS gradient polyacrylamide gel. After electrophoresis, a Western blot was performed using nitrocellulose as the transfer media. Murine anti-FLAG primary monoclonal antibody M1 (IBI/Kodak Scientific Imaging Systems) was used at a concentration of 2.5  $\mu$ g/mL. The secondary antibody was an alkaline phosphatase

conjugated, goat anti-mouse IgG F(ab')<sub>2</sub> (Boehringer Mannheim Corp.). The blot was developed using an Alkaline Phosphatase Conjugate Substrate Kit (Bio-Rad, #170-6432). In the non-reduced periplasm samples, sharp immunoreactive bands were observed at ~17,000 daltons, ~30,000 daltons and another at a molecular weight > 43,000 daltons. A single band migrating at ~17,000 daltons was observed in these same samples when run under reducing conditions. Similar results were observed with the whole cell samples but with higher background. The M1 antibody only detects the FLAG epitope if it has a free N-terminus; therefore, the antibody only reacts with the expressed protein transported to the periplasm where the ompA signal sequence has been removed.

To compare expression levels in different *E. coli* strains, pFLAGROB#5 was used to transform competent BL21 (Novagen, Inc.), W3110 (ATCC No. 27325) and JM105 (Pharmacia) cells. FLAG/rat *ob* protein expression was compared to the original DH5αF' strain by Western Blot analysis, as outlined above. W3110 and JM105 produced approximately five to ten times more protein than either the BL21 or DH5αF' strains. Both the ~17,000 dalton and ~30,000 dalton immunoreactive bands were observed under non-reducing conditions from all of the strains tested.

#### Example 5 -- Rat *ob* Gene Fermentation Expression

Quantities of rat *ob* gene product or rat *ob* gene fusion product can be produced using the below described methods for the production of FLAG/rat *ob* gene product.

FLAG/rat *ob* gene product was produced via 10 and 60 liter fermentations. The below description refers to a 10 liter fermentation. Prior to inoculation, the production tank was prepared with 10 liters of Luria broth (Tryptone (Difco), 10

g/L; NaCl (VWR), 10 g/L; Yeast extract (Marcor), 5 g/L; glucose (Atlas Clintose), 4 g/L; polypropyleneglycol as antifoam, 0.25 g/L. The Luria broth was steam sterilized in place at 121°C for 30 minutes and then allowed to cool (typically 24 hours) before fermentation commenced. Fermentation conditions were as follows:

5 200 mL of Luria broth was inoculated with *E. coli* strain W3110, which had been previously transformed with the expression vector pFLAGROB#5, and grown overnight in an incubated shaker (37 C, 240 rpm). Prior to inoculation, 0.05 g/L of ampicillin was added to the fermentor for plasmid selection. The inoculum was transferred to the production tank after approximately 17 hours in a volume ratio of

10 1:100. Typically, the inoculum optical density had exceeded 1 OD at 600 nm ( $OD_{600}$ ). The fermentation was controlled at the following set points: Temperature at 37 C; dissolved  $O_2$  at 20% of saturation at 37°C, 5 psi; pH at 6.8; airflow rate at 5 Standard Liters per minute.

Fermentation was monitored at least every hour for the following parameters:

15 temperature, agitation rate (rpm), pH, dissolved  $O_2$  (%), airflow rate (SLPM), pressure (psi), offgas  $O_2$  (%), offgas  $CO_2$  (%),  $OD_{600}$ , and glucose concentration (g/L). When the fermentation reached an  $OD_{600}$  of approximately 3, the *E. coli* were induced to produce FLAG/*ob* gene product with .238 g/L IPTG (Isopropyl- $\beta$ -D-thiogalactopyranoside; Mannheim Gmbh, Lot 13951720-98). Two hours after

20 induction of the fermentation, the cells were harvested via centrifugation in 1 liter Nalgene bottles. The broth was decanted away, and the cells were frozen by immersion in liquid nitrogen.

**Example 6 -- Affinity Purification of FLAG/rat *ob* Fusion Protein**

A 250 mL pellet obtained from the *E. coli* shake flask fermentation described in Example 4 was resuspended in 50 mL of extraction buffer A (50 mM Tris (pH = 8.0), 5 mM EDTA, 0.25 mg/mL lysozyme (Sigma, L-6876), 50 µg/mL sodium azide, 250 µl of 100 mM AEBSF/Ethanol) and the suspension processed with a Dounce homogenizer. The whole was incubated at room temperature for 15 minutes followed by addition of 5 mL of extraction buffer B (1.5 M NaCl, 100 mM CaCl<sub>2</sub>, 100 mM MgCl<sub>2</sub>, 50 µg/mL Ovomucoid protease inhibitor (Sigma, T-9253), 0.02 mg/mL DNase I (Sigma, D-4527)). After centrifugation (25,000 xg), Beckman J2-21 centrifuge, J-20 rotor, 14,300 rpm) for one hour at 4°C, the supernatant was applied to an anti-FLAG M1 affinity column prepared in accordance with manufacturer's instructions and cycled three times. The column was washed with 3 x 6 mL of 2 mM CaCl<sub>2</sub>/TBS (50 mM Tris/150 mM NaCl) to remove all non-FLAG proteins. The FLAG proteins were eluted with 4 mM EDTA/TBS to give a mixture of monomer and dimer (identified by SDS-PAGE, Western blotting) and two lower molecular weight fractions which were noted on reverse phase HPLC analysis.

Further purification of the FLAG-*ob* protein was accomplished by gel filtration using a Pharmacia Superose 12 HR10/30 column. Approximately 4 mL of Flag-*ob* protein in EDTA/TBS (50mM Tris/150mM NaCl/4mM EDTA pH 7.4) was concentrated by speedvac on medium heat in two separate 12 x 25 mm Nunc Lo-Sorp test tubes, yielding approximately a 3 to 1 concentration in about two hours. 1.3 mL of concentrated protein was injected onto a Superose 12 HR10/12 column and the column run uphill at 0.25 mL/min using an Automated FPLC

System from Pharmacia Biotech. The running buffer was 50 mM Tris/150 mM NaCl (pH 7.4). Fractions were collected at one minute intervals. Monomer and dimer fractions were collected and were shown to be about 95% pure by SDS-PAGE with silver stain detection.

5       **Example 7 – Additional Purification of FLAG/rat *ob* Fusion Protein by Ion Exchange Chromatography**

The initial affinity chromatography described in Example 6 was complicated by antibody binding interference from a lower molecular weight 13 mer containing the FLAG sequence (DYKDDDDKVPHIK). Q-Sepharose resins were determined  
10 to be efficacious in purifying crude FLAG-*ob* and in separating FLAG-*ob* monomer from dimer protein. In particular, a Q-Sepharose HP column was used to fractionate affinity column flow-through and to yield highly active partially purified dimer.

All ion exchange purification was done at 4°C using an Automated FPLC System from Pharmacia Biotech equipped with a Conductivity Monitor. Elution  
15 profile data were collected and analyzed using the FPLC manager software package running on an NEC Powermate 466 computer. FLAG-*ob* protein was detected by the following methods: absorbance detection at 280 nm, SDS-PAGE with detection using Coomassie Brilliant Blue, silver staining, or Western Blotting. Reagents, 10-20% Tricine gels and gel apparatus from NOVEX were used for SDS-PAGE. Silver  
20 staining was done using a silver stain kit from Accurate Chemical Company and Western Blotting was done as previously described in Example 4, but using PVDF membranes instead of nitro-cellulose. Sequencing was done using ABI model 470A or 476A protein sequencers using standard protocols.

200 mL of FLAG affinity column flow-through material, which contained both monomeric and dimeric FLAG-*ob* proteins (as determined by Western Blotting), was subjected to anion exchange chromatography. The material was first clarified by filtration using a 0.65µm Durapore membrane filter (Millipore), and concentrated using a 200 mL Amicon microfiltration unit equipped with a YM3 membrane. The concentrated material was then diluted to 50 mL with 20 mM Tris HCl, pH 7.4 and loaded onto a HiLoad 16/10 Q-Sepharose HP column (Pharmacia Biotech) using a flow rate of 3.0 mL/min. The column was eluted at 3.0 mL/min. using a 430 mL gradient to 20 mM Tris HCl pH 7.4 containing 1.0M NaCl while collecting 6.0 mL fractions. Fractions from this run were analyzed by SDS-PAGE with detection using Coomassie Brilliant Blue staining and Western Blotting. The gel results indicated that monomeric FLAG-*ob* protein eluted mainly in earlier fractions and that dimeric FLAG-*ob* protein eluted in middle fractions. One smaller form was observed at an apparent molecular weight of 10 kD in later fractions.

Another 100 mL of FLAG affinity column flow-through material was subjected to anion exchange chromatography. The flow-through material was concentrated to 23 mL using a 200 mL Amicon microfiltration unit equipped with a YM3 membrane. The concentrated material was then diluted to 40 mL with 20 mM Tris HCl pH 7.4 and loaded onto a HiLoad 16/10 Q-Sepharose HP column (Pharmacia Biotech) using a flow rate of 3.0 mL/min. The column was eluted at 3.0 mL/min. using a 430 mL gradient to 20 mM Tris HCl pH 7.4 containing 1.0 M NaCl while collecting 6.0 mL fractions. Fractions from this run were analyzed by SDS-PAGE with detection using Coomassie Brilliant Blue staining and Western Blotting.

Again, the gel results indicated that monomeric FLAG-*ob* protein eluted mainly in earlier fractions and that dimeric FLAG-*ob* protein eluted in later fractions.

200 mL of FLAG affinity column flow-through material was subjected to cation exchange chromatography. The flow-through was diluted with water and brought to pH 4.5 by the careful addition of dilute acetic acid, final volume 4L, and batch absorbed overnight to 50 mL of SP-Sepharose Fast-Flow resin (Pharmacia Biotech). The resin was poured into an XK 26/10 column and washed with 25 mM sodium acetate pH 4.5. The column was eluted using a 180 mL gradient to 25 mM sodium acetate pH 4.5 containing 0.5M NaCl using a flow rate of 6.0 mL/min. 12 mL fractions were collected into tubes containing 2 mL of 0.25M Tris HCl pH 8.0 buffer to give a final pH of about 7.5 for each fraction. Fractions from this run were analyzed using SDS-PAGE with detection using Coomassie Brilliant Blue staining and Western Blotting. The gel results indicated that monomeric FLAG-*ob* protein eluted most strongly in fractions 7-9 and that minor amount of dimeric FLAG-*ob* protein eluted in fractions 6-8.

A further approach was undertaken to enhance the purity of active FLAG-*ob* dimer proteins and *ob* monomer fusion proteins isolated as described above. 1 mL of a Q-Sepharose dimer-containing fraction derived from Q-Sepharose purification of a cell pellet from a 60 L fermentor run described in Example 5 was brought to 10 mM  $\text{CaCl}_2$  by the addition of 1M  $\text{CaCl}_2$ . This material was then applied to an 0.5 mL bed volume Anti-FLAG M1 affinity column and washed with 20 column volumes of TBS containing 10 mM  $\text{CaCl}_2$ . The Anti-FLAG affinity column was then eluted using 20 column volumes of 50 mM Tris containing 25 mM EGTA pH 8.2, followed by elution using 100 mM glycine HCl buffer pH 3.0. Fractions were

analyzed by SDS-PAGE using silver staining and Western blotting for detection. It was found that the majority of the FLAG-*ob* protein eluted from the column in the EGTA step, and that only a trace of material remained to be eluted during the glycine HCl step. Furthermore, it was determined that this method yielded  
 5 approximately 90% pure FLAG-*ob* dimer protein.

#### **Example 8 -- Preparation of Anti-*ob* monomer and Anti-*ob* dimer Antibodies**

Anti-*ob* monomer antibodies, anti-*ob* monomer fusion protein antibodies, anti-*ob* dimer antibodies, and/or anti-*ob* dimer fusion protein antibodies in accordance with the present invention are prepared as follows. A sample of *ob*  
 10 monomer or dimer, such as one of those prepared by the methods of Examples 5-7, 11, 14, 20, 22 and 26, and tested as described in Example 23, or a synthetic *ob* protein fragment, is conjugated to bovine thyroglobulin (THY, Sigma) as a carrier protein using a 2:1 ratio of carrier to peptide and glutaraldehyde or sulfosuccinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylate (sulfo-SMCC)  
 15 as a heterobifunctional linker to facilitate conjugation. Examples of synthetic *ob* protein fragments include the following modified fragments of the mouse *ob* sequence: (1) VPIQKVQDDTKTGCG-NH<sub>2</sub>; (2) Acetyl-SNDLENLRDLLHGCG-NH<sub>2</sub>; (3) Acetyl-CSLPQTSGLQKPESLDG-NH<sub>2</sub>; (4) Acetyl-GCGSLQDILQQLDVSPEA-OH; (5) Acetyl-GCGLSKMDQTLAVYQ-  
 20 VLTSLPSQNVLQIANDLENLRD-NH<sub>2</sub>; and, (6) VPIQKVQDDTKTLIKTIVTRINDISHTQSVG-CG-NH<sub>2</sub>. Examples of synthetic fragments of the human *ob* sequence include the following modified fragments: (1) Ac-98-109-GCG- NH<sub>2</sub>; (2) Ac-G-117-136- NH<sub>2</sub>; and (3) 149-167-NH<sub>2</sub>. Examples of synthetic fragments of the rat *ob* sequence include the following: (1) Ac-148-167



and (2) GCG-52-71-NH<sub>2</sub>. In addition a fragment generated by CNBr cleavage of rat ob protein (74-167) may also be used for immunization. A 10 mg/mL solution of carrier protein in 50 mM sodium borate buffer, pH 7.4 is made to which sulfo-SMCC (Pierce, Rockford, IL) is added to a final concentration of 6 mg/mL. This mixture is incubated at room temperature for 1-2 hours and then dialyzed against water overnight at 4°C to remove free SMCC. Peptide is dissolved at 1 mg/mL in HPLC grade water and activated carrier protein added dropwise with stirring and the pH adjusted as necessary to prevent precipitation of the peptide. The mixture is incubated with stirring at room temperature for 3 hours and then dialyzed against water overnight at 4°C. Immunization using DNA constructs of either the human or rat ob sequence inserted into a mammalian expression vector, preferably pcDNA3 can also be prepared by injecting DNA, preferably at 100 µg/ml, in the quadriceps muscle of the mouse, and have been successfully used to prepare anti-ob antibodies.

Glutaraldehyde conjugations were performed by mixing peptide at 2 mg/mL in HPLC grade water with thyroglobulin made up in borate buffer as above at a 2:1 ratio of carrier protein to peptide. Glutaraldehyde is added to a final concentration of 0.1%, the pH is adjusted as necessary to prevent precipitation of the peptide, and the mixture is stirred at room temperature for 3 hours. The mixture is then dialyzed at 4°C overnight against water.

Preferably, the Balb/C (H-2D) and ND4 strains of mice (Harlan Sprague Dawley; San Diego, CA) are used for immunizations. All animals are typically about 8-12 weeks of age when immunization protocols are initiated. Animals are primed intraperitoneally with 50 µg of antigen emulsified in Freund's Complete Adjuvant (Sigma). At three week intervals the animals are boosted intraperitoneally

with 50 µg of antigen emulsified in monophosphoryl lipid A, trehalose dimycolate (MPL+TDM RAS) adjuvant (Ribi Immunochem Research, INC., Hamilton, MT). Animals are bled 7 days after the third injection and the peptide-specific antibody measured by a solid-phase RIA, such as described below. Further boosts and bleeds  
5 are performed as needed until a sufficient antibody titer is obtained.

Antisera are assayed by a solid-phase radioimmunoassay. Dynatech Removawell Immulon 2 plates (Dynatech, Chantilly, VA) are coated with 50 µl/well goat anti-mouse IgG+IgM antibody (Accurate, Westbury, NY) diluted in 0.05 M sodium carbonate buffer, pH 9.5 to a dilution of 20 µg/mL. The antibody is allowed  
10 to passively adsorb overnight at 4°C. The plates are washed extensively with PBS+0.1% Tween 20 and water, blocked with 100 µl/well of 1% nonfat dry milk in sodium carbonate buffer for 1-2 hours, and washed again. Antisera are serially diluted in hybridoma growth medium, RPMI 1640 (Irvine Scientific, Santa Ana, CA) + 10% fetal bovine serum (Sigma) and 50 µl/well added to the plates and  
15 incubated for 2 hrs. at room temperature. The plates are washed a second time, and radiolabelled recombinant *ob* protein or the immunizing synthetic *ob* protein fragment conjugated to BSA diluted in RIA buffer to 30,000 cpm/100 µl is added and incubated at room temperature for 3 hours. RIA buffer consists of PBS, pH 7.4 with 0.1% Triton X-100, 0.1% teleostean gelatin (Sigma), 0.01% sodium azide, and  
20 0.001% thimerosal. Following a final wash, the wells are separated and placed in 12 X 75 mm polystyrene tubes and counted, for example in a LKB Gammamaster 1277 gamma counter.

All cell lines are maintained in RPMI 1640 medium supplemented with 10% FBS and 3% Origen Hybridoma Cloning Factor (HCF, Fisher Scientific, Tustin,

CA) and incubated at 37°C in 95% air/5% CO<sub>2</sub>. Hybridomas are selected in growth medium supplemented with hypoxanthine-aminopterin-thymidine (HAT) (Boehringer Mannheim (Indianapolis, IN), as described in Littlefield, J. W., (1964) *Science* 145: 709.

5        Three days after a final 50 µg intravenous boost, animals are sacrificed and spleens removed. The spleens are teased apart and the splenocytes fused to P3X63-Ag8.653 myeloma cells (Kearney et al., (1979) *J. Immun.* 123: 1548) at a ratio of 4:1. Polyethylene glycol 1,500 MW (Aldrich, Milwaukee, WI) at a concentration of 35% is used following a modification of the method of Gerhard (1980), in Kennet et  
10 al. (Eds.) *Monoclonal Antibodies*, page 370 (Plenum Press). Cells are plated at about  $1.5 \times 10^5$  cells/well in HAT selective medium in 96-well microtiter plates and incubated for 10-14 days before the supernatants are screened for specific antibody.

Selected hybridoma cell lines are subcloned by limiting dilution, and antibodies raised in ascites in either Balb/C or nude mice.

15        **Example 9 – Vector Construct for FLAG-rat *ob* Periplasmic Expression**

In order to prepare a vector construct suitable for periplasmic expression in *E. coli* BL21(DE3) cells, pFLAGROB#5 plasmid DNA (described in Example 4 above) was digested with restriction endonucleases *Nde*I and *Eco*RI (Boehringer Mannheim Corp.) and the resulting fragments were run on a 1% agarose gel. The  
20 smaller fragment, approximately 540bp, containing the ompA signal and mature rat *ob* coding region, was purified by running the DNA onto DE-81 paper and subsequently eluting with 1M NaCl. The isolated DNA fragment was ethanol precipitated, dried and resuspended in sterile distilled water. Likewise, pET27b+ vector DNA was purchased from Novagen Inc. (Madison, WI), digested with *Nde*I

and *EcoRI* and run on a 1% agarose gel. The vector DNA fragment was excised from the gel and isolated using a silica membrane system (SpinBind® DNA Recovery System; FMC BioProducts, Rockland, ME) following the manufacturer's protocol. The isolated insert DNA was ligated to the vector DNA followed by  
5 transformation into competent *E. coli* BL21 cells (Novagen Inc.; Madison, WI). Transformants were screened by isolating plasmid DNA using an alkaline lysate method (Wizard® Minipreps DNA Purification System, Promega, Madison, WI) followed by restriction endonuclease digestion with *NdeI* and *EcoRI* to check for the presence of the *ompA/ratob* insert. A positive clone, pET27ROB, was selected and  
10 sequenced. pET27ROB DNA was used to transform competent *E. coli* BL21(DE3) (Novagen Inc., Madison WI) for expression of FLAG-rat *ob* protein.

#### **Example 10 – Periplasmic expression of FLAG-rat *ob* protein**

In order to evaluate methods for enhanced periplasmic production of *ob* fusion proteins, a seed culture of pET27ROB from Example 9 was inoculated from a  
15 previously prepared plate and grown overnight at 37°C in 150mL of 20g/L LB media (Gibco BRL, Gaithersburg, MD) containing 50mg/L kanamycin (Fisher Biotech, Fair Lawn, NJ) to a final OD<sub>600nm</sub> of 3.46. Six 2L Fernbach flasks containing 1L each of sterile filtered 25g/L LB media (Gibco BRL, Gaithersburg, MD) containing 50mg/L kanamycin were inoculated with 15mL of the overnight  
20 seed culture. The six flasks were then grown at 27°C for 4 hours to an OD<sub>600nm</sub> of 0.424 in the presence of 5g/L glucose and production of FLAG-rat *ob* protein was induced by the addition of IPTG (isopropyl, thio β-G-galactosidase; Fisher Biotech, Fair Lawn NJ) to a final concentration of 0.5mM. After induction, the cells were incubated for 3h at 28°C to a final OD<sub>600nm</sub> of 1.25 and harvested by centrifugation in

a JA10 rotor for 15 min. at 5000rpm using a model J2-21 centrifuge (Beckman Instruments Inc., Fullerton, CA). The 13.4g cell pellet obtained in this manner was then washed three times by resuspending the pellet to 25g/L with 10mM Tris buffer, pH 8.0 and centrifuging the cells at 5000rpm for 10 min. in a Beckman JA-10 rotor.

5 The washed cell pellet was then resuspended to 25g/L with 30mM Tris buffer, pH 8.0 containing 0.5M sucrose and 1mM EDTA and incubated at room temperature for 10min. to allow the cells to equilibrate. The cells were then removed from the sucrose-containing buffer by centrifugation, as above, and shocked by resuspension to 40g/L with ice-cold water containing 1mM Peflabloc SC (Centerchem, Stamford,

10 CT). The periplasmic extract obtained in this manner was clarified by centrifugation at 8500rpm for 10 min. in a JA-10 rotor using a model J2-21 centrifuge (Beckman Instruments Inc., Fullerton, CA) and stored at 4C until further purification. The yield of FLAG-rat *ob* protein from this protocol was 41mg by HPLC analysis, nearly 7 mg/L of cells.

15 **Example 11 --Purification of FLAG-Rat *ob* protein**

FLAG-rat *ob* protein prepared as described in Example 10 was readied for purification by adding Tween-80 to a final concentration of 0.02%w/v and adjusting the pH of the solution to 8.5 by the careful addition of 100mM BisTris-propane free base in water. The protein solution was then clarified by filtration through an

20 0.45µm Sartobran filter (Sartorius Corp., Tustin, CA). The clarified solution was loaded onto DE-52 resin (Whatman Inc., Clifton, NJ) packed into an XK16 column (Pharmacia Biotech, Piscataway, NJ) to a bed height of 6.25cm at a flow rate of 4mL/min. The column was washed with 17mM BisTris-propane containing 0.02% Tween-80 at pH 8.5 and the rat *ob* protein was eluted using a linear gradient to 0.5M

NaCl in 17mM BisTris-propane buffer containing 0.02% Tween-80 at pH 8.5. Protein elution was monitored using absorbance detection at 280nm and verified by SDS-PAGE and RP-HPLC analysis of fractions collected during the elution gradient. Appropriate fractions were pooled, brought to pH 2.5 by the addition of  
5 TFA to 0.3% v/v and loaded onto a VYDAC 218TP1022 HPLC column (Vydac, Hesperia, CA) using a flow rate of 15mL/min. The rat *ob* protein was eluted from the column using a linear gradient from 0.1%TFA in 45% acetonitrile to 0.1% TFA in 60% acetonitrile over 15 minutes. The elution of FLAG-rat *ob* protein was detected by absorbance at 214nm and confirmed by SDS-PAGE. Monomeric  
10 FLAG-rat *ob* protein and dimeric FLAG-rat *ob* protein were pooled separately, concentrated by evaporation using a Speedvac Plus SC110A (Savant, Farmingdale, NY), flash frozen and lyophilized as TFA salts using a FREEZE DRYER 4.5 (LABCONCO, Kansas City, MO). The proteins obtained in this manner were ≥95% pure by SDS-PAGE analysis. Monomeric and dimeric FLAG-rat *ob* proteins were  
15 tested and shown to be active *in vivo* as described in Example 23.

#### Example 12 -- Vector Construct for Rat *ob* Periplasmic Expression

In order to prepare a vector construct suitable for periplasmic expression of unfused rat *ob* proteins in BL21(DE3) *E. coli* cells, the coding region for mature rat *ob*, Val<sup>22</sup> to Cys<sup>146</sup>, was PCR amplified from a cloning vector using reagents from  
20 the Perkin Elmer PCR kit (Perkin Elmer, Foster City, CA). The first 18 bases of the upstream primer, 5'-GCT ACC GTT GCG CAA GCT GTG CCT ATC CAC AAA GTC CAG G, are homologous to the coding sequence of the last 18 bases of the *ompA* leader sequence. The remaining bases are homologous to the first 22 bases of the coding region for mature rat *ob*. The downstream primer used for the PCR was a

vector specific, T7 terminator primer found in the pET series of expression vectors (Novagen, Madison, WI). PCR amplification was performed in a 100µl volume containing 30ng of template DNA, 0.5µM of each primer, 2.5 units of AmpliTaq DNA<sup>®</sup> Polymerase, 10mM Tris-HCl, pH 8.3, 50mM KCl, 0.2mM each dNTP, and 2.5mM MgCl<sub>2</sub>. The reaction was incubated for two cycles at 95°C/1 min, 65°C/1 min, and 72°C/1min followed by 30 cycles of 95°C/30 sec, 65°C/30 sec, 72°C/30sec followed by a final incubation of 10 min at 72°C. In a separate PCR reaction, the ompA leader sequence was amplified from the pFLAGROB#5 vector of Example 4 using, as the upstream primer, the pFLAG vector specific, N26 primer. The first 18 bases of the downstream primer, 5'- GAC TTT GTG GAT AGG CAC AGC TTG CGC AAC GGT AGC GAA AC, correspond to the sequence of the non-coding strand of the first 18 bases of mature rat *ob*. The remaining 23 bases of the downstream primer correspond to the sequence of the non-coding strand of the last 23 bases of the ompA leader sequence. The PCR reaction conditions and cycling times were set up exactly as for the previous reaction with the only difference being the annealing temperature, which was 53°C for this reaction. A secondary PCR reaction was performed in a 100µl volume containing equimolar concentrations of the two primary PCR fragments along with the N26 and T7 terminator primers following the PCR procedure outlined above. The annealing temperature in the cycling profile was 65°C. The resulting PCR product is a fusion of the ompA leader sequence with the mature rat *ob* coding sequence. The PCR fragment was purified using the QIAquick PCR Purification kit (QIAGEN Inc.) following the kit protocol, digested with *Nde*I and *Eco*RI, and run on a 1% agarose gel. The digested fragment was isolated from the gel and purified using a silica membrane system (SpinBind<sup>®</sup>

DNA Recovery System; FMC BioProducts, Rockland, ME) following the manufacturer's protocol. The fragment was ligated into the pET27b+ vector also cut with *NdeI* and *EcoRI*. The resulting subclone, pET27ROB(-), was sequenced to confirm proper construction of the coding region.

5                   **Example 13 -- Periplasmic expression of Rat *ob* protein**

In order to evaluate methods for enhanced periplasmic production of unfused *ob* proteins, a seed culture of pET27ROB(-) was inoculated from a frozen glycerol stock and grown overnight at 37°C in 150mL of 20g/L LB media (Gibco BRL, Gaithersburg, MD) containing 50mg/L kanamycin (Fisher Biotech, Fair Lawn NJ) to  
10 a final OD<sub>600nm</sub> of 3.5. Six 2L Fernbach flasks containing 1L each of sterile filtered 25g/L LB media (Gibco BRL, Gaithersburg, MD) containing 5g/L glucose and 50mg/L kanamycin were inoculated with 15mL of the overnight seed culture. The six flasks were then grown at 30°C for 2.5 hours to an OD<sub>600nm</sub> of 0.49 and production of rat *ob* protein was induced by the addition of IPTG (isopropyl, thio β-  
15 G-galactosidase; Fisher Biotech, Fair Lawn NJ) to a final concentration of 0.5mM. After induction, the cells were incubated for 3h at 30°C to a final OD<sub>600nm</sub> of 1.53 and harvested by centrifugation in a JA10 rotor for 15 min. at 5000rpm using a model J2-21 centrifuge (Beckman Instruments Inc., Fullerton, CA). The 20g cell pellet obtained in this manner was then washed three times by resuspending the  
20 pellet with 800mL of 10mM Tris buffer, pH 8.0 and centrifuging the cells at 5000rpm for 10 min. in a Beckman JA-10 rotor. The washed cell pellet was then resuspended in 800mL of 30mM Tris buffer, pH 8.0 containing 0.5M sucrose and 1mM EDTA and incubated at room temperature for 10minutes to allow the cells to equilibrate. The cells were then removed from the sucrose-containing buffer by



centrifugation, as above, and shocked by resuspension in 500mL of ice-cold water containing 1mM Peflabloc. The periplasmic extract obtained in this manner was clarified by centrifugation at 8500rpm for 10 minutes in a Beckman JA-10 rotor and stored at 4°C until further purification. The yield of rat *ob* protein from this protocol  
5 was 40.6mg by HPLC analysis, nearly 7 mg/L of cells.

#### Example 14 – Purification of Rat *ob* protein

Rat *ob* protein prepared as described in Example 13 was readied for purification by adding Tween-80 to a final concentration of 0.02%w/v and adjusting the pH of the solution to 8.5 by the careful addition of 100mM BisTris-propane free  
10 base in water. The protein solution was then clarified by centrifugation at 13,000xg for 25min., and filtration through a 5µm Millex-SV filter (Millipore, ) followed by filtration through an 0.45µm Sterivex-HV filter. The clarified solution was loaded onto a 14mL Q-Sepharose HP column (Pharmacia Biotech, ) at a flow rate of 6mL/min. The column was then washed with 17mM BisTris-propane containing  
15 0.02% Tween-80 at pH 8.5 and the rat *ob* protein was eluted using a linear gradient to 0.17M NaCl in 17mM BisTris-propane buffer containing 0.02% Tween-80 at pH 8.5. Protein elution was monitored using absorbance detection at 280nm and verified by SDS-PAGE and RP-HPLC analysis of fractions collected during the elution gradient. Appropriate fractions were pooled, brought to pH 2 by the addition  
20 of TFA to 0.2% v/v and loaded onto a VYDAC 218TP1022 HPLC column (Vydac, Hesperia, CA) using a flow rate of 15mL/min. The rat *ob* protein was eluted from the column using a linear gradient from 0.1%TFA in 45% acetonitrile to 0.1% TFA in 60% acetonitrile over 15 minutes. The elution of rat *ob* protein was detected by absorbance at 214nm and confirmed by SDS-PAGE. Monomeric rat *ob* protein and

dimeric rat *ob* protein were pooled separately, concentrated by evaporation using a Speedvac Plus SC110A (Savant, Farmingdale, NY), flash frozen and lyophilized as a TFA salt using a FREEZE DRYER 4.5 (LABCONCO, Kansas City, MO). The proteins obtained in this manner was  $\geq 95\%$  pure by SDS-PAGE analysis.

- 5 Monomeric rat *ob* protein prepared as described herein was tested and shown to be active *in vivo* as described in Example 23.

#### Example 15 – Vector Construct for Met-rat *ob* Intracellular Expression

A vector construct containing a modified rat *ob* coding sequence was prepared to evaluate its utility for intracellular protein expression. The coding  
10 region for mature rat *ob*, Val<sup>22</sup> to Cys<sup>146</sup>, was PCR amplified from a cloning vector using reagents from the Perkin Elmer PCR kit (Perkin Elmer, Foster City, CA). The upstream primer, 5'-TATACAT ATG GTT CCG ATC CAC AAA GTC CAG GAT GAC, incorporated an *Nde*I site (underlined) onto the 5' end of the PCR fragment to facilitate cloning. The *Nde*I site also has within it the initiating methionine codon,  
15 ATG. The codons for Val<sup>22</sup> (GTT) and Pro<sup>23</sup> (CCG) of the rat *ob* protein were changed from the original DNA sequence to preferred codons for *E. coli* expression. The downstream primer used for PCR was a vector (pET15b, Novagen Inc, Madison, WI) specific, T7 terminator primer. PCR amplification was performed in a 100 $\mu$ l volume containing 50ng of pET15ROB (a construct containing a T7  
20 promoter and the native codons for the Met-rat *ob* sequence), 0.5 $\mu$ M each primer, 2.5 units of AmpliTaq DNA<sup>®</sup> Polymerase, 10mM Tris-HCl, pH 8.3, 50mM KCl, 0.2mM each dNTP, and 2.5mM MgCl<sub>2</sub>. The reaction was incubated for 2 cycles at 95°C/1 min, 53°C/1 min, and 72°C/1 min followed by 30 cycles at 95°C/30 sec, 53°C/30sec, and 72°C/30 sec followed by a final incubation of 10 min at 72°C.

Analytical agarose gel electrophoresis confirmed a single band of the expected size, approximately 540bp. The remaining PCR reaction material was purified using the QIAquick PCR Purification kit (QIAGEN Inc.) following the kit protocol. The PCR fragment was digested with *NdeI* and *XhoI* and cloned into the pET27b+ vector  
5 (Novagen Inc, Madison WI) previously digested with the same enzymes and treated with Calf Intestinal Alkaline Phosphatase (Boehringer Mannheim Corp.). The resulting subclone, pET27OPTII #4, was sequenced using rat *ob* specific primers to confirm proper construction of the coding region. pET27OPTII DNA was used to transform competent *E. coli* BL21(DE3) for expression of the Met-rat *ob* protein.

10     **Example 16 -- Intracellular Expression of Met-rat *ob* in Inclusion Bodies**

In order to evaluate methods for enhanced intracellular production of unfused *ob* proteins, including *ob* proteins having an N-terminal methionine, a seed culture of pET27OPTII was inoculated from a frozen glycerol stock and grown overnight at 37°C in 150mL of 20g/L LB media (Gibco BRL, Gaithersburg, MD) containing  
15 50mg/L kanamycin (Fisher Biotech, Fair Lawn NJ) to a final OD<sub>600nm</sub> of 3.86. Six 2L Fernbach flasks containing 1L each of 25g/L LB media containing 50mg/L kanamycin were inoculated with 20mL of the of the overnight seed culture, grown at 35°C for 2.5 hours (OD<sub>600</sub> = 0.723) and production of Met-rat *ob* protein was induced by the addition of IPTG (isopropyl, thio β-G-galactosidase; Fisher Biotech,  
20 Fair Lawn NJ) to a final concentration of 1mM. After induction, the cells were incubated for 5.5h at 36°C and harvested by centrifugation in a JA10 rotor for 15 min. at 5000rpm using a model J2-21 centrifuge (Beckman Instruments Inc., Fullerton, CA). The 18.1g cell pellet was flash frozen and stored at -20°C. The cell pellet was thawed and resuspended in lysis buffer (500mL of 100mM potassium

phosphate, pH 6.5) and lysed by 2 passages through a model 110Y microfluidizer (Microfluidics Corp., Newton, MA). The inclusion bodies from the lysed cells were collected by centrifugation at 5000xg for 25 minutes at 4°C using an RC-3B centrifuge (Dupont Co., Willmington, DE). Met-rat *ob* protein-containing inclusion  
5 bodies were washed by resuspending with 500mL of 100mM potassium phosphate, pH 6.5 and collected by centrifugation as above. The washed inclusion bodies were frozen and stored at -80°C. The typical yield was 1.8g of Met-rat *ob* protein per 6L of cultured cells.

10      **Example 17 – Solubilization and Refolding of Met-rat *ob* Protein from Inclusion Bodies to Generate Both Monomeric and Dimeric Protein Forms**

In order to generate quantities of properly folded dimeric as well as monomeric *ob* protein suitable for further purification, the phosphate-washed inclusion bodies prepared as described in Example 16 were thawed and dissolved in  
15 50mM ammonium bicarbonate containing 8M urea at 6mg of wet inclusion body material per mL of solubilization buffer. The solubilized protein (360mL) was dialyzed at 4°C overnight against 10L of 17mM Bis-Tris propane containing 0.02% Tween-80 at pH 8.5 using SpectraPor regenerated cellulose membrane with a stated molecular weight cutoff of 3500 daltons. Reversed-phase HPLC analysis showed  
20 that the protein in the initial urea containing solubilization buffer contained 1-4% dimer as a percent of the total *ob* protein, while the dialyzed material contained 23% dimer, thus confirming the desired conversion of *ob* monomer to *ob* dimer form. Proper folding was demonstrated by *in vivo* activity of the isolated purified proteins, as described in Example 23.

25      **Example 18 – Cellulose-based Anion Exchange Purification of Refolded Met-rat *ob* Protein**

This experiment shows that purification of *ob* proteins, including *ob* monomer, *ob* fusion monomer, *ob* dimer and *ob* fusion dimer, using cellulose-based resin results in surprisingly high yield recovery of protein. DE-52 resin (Whatman  
5 Inc., Clifton, NJ) was swollen in 17mM BisTris-propane containing 1M sodium chloride at pH 8.4 and packed into a Pharmacia Biotech XK16 column to a packed bed height of 5.6cm. The Met-rat *ob* protein solution (271mg monomer and 76mg dimer in 260mL) was loaded onto the column using a flow rate of 4mL/min. The Met-rat *ob* protein was eluted using a linear gradient from 17mM BisTris-propane  
10 containing 0.02% Tween-80 at pH 8.5 to 17.5mM Bis-Tris propane containing 0.5M NaCl and 0.02% Tween-80 at pH 8.5. Protein elution was monitored using absorbance detection at 280nm and verified by SDS-PAGE and RP-HPLC analysis of fractions collected during the elution gradient. Appropriate fractions were pooled to give an average 70% yield of purified monomeric and dimeric Met-rat *ob* protein.

15      **Example 19 – Separation and Further Purification of Met-rat *ob* Protein Monomer and Dimer with Reversed Phase-HPLC**

Pooled anion exchange fractions prepared as described in Example 18 were carefully acidified to a final concentration of 0.3% TFA using a 10% solution of  
20 TFA in water. The acidified protein fractions were loaded onto a Vydac 218TP1022 HPLC column (Vydac, Hesperia, CA) at a flow rate of 15mL/minute and eluted using a linear gradient from 0.1% TFA in 45% acetonitrile to 0.1% TFA in 65% acetonitrile over 20 minutes. Protein elution was monitored by absorbance at 214nm and appropriate fractions were pooled and concentrated to remove acetonitrile by  
25 evaporation using a Speedvac Plus SC110A (Savant, Farmingdale, NY). Using this technique, monomer was separated from dimer and trace remaining endotoxin was

removed from the preparation (to a level less than 1 EU/mg). When the preparation of the TFA salt of the protein was desired, the concentrated HPLC pool was flash-frozen and lyophilized on a FREEZE DRYER 4.5 (LABCONCO, Kansas City, MO).

5      **Example 20 -- *Ob* Protein as a Lyophilized Ammonium Bicarbonate Salt**

Monomeric or dimeric *ob* proteins may be prepared as lyophilized ammonium bicarbonate salts. In this experiment, solutions of monomeric and dimeric *ob* protein prepared as described in Example 19, or the TFA salt form of  
10 these proteins dissolved in 0.1% TFA in water, were brought to pH 7 by the addition of concentrated ammonium bicarbonate, transferred to SpectraPor 3500MW cutoff dialysis tubing and fully dialyzed vs. 20mM ammonium bicarbonate (pH 7.8 when freshly dissolved) at 4°C. The dialyzed protein solutions were flash frozen and lyophilized to dryness using a FREEZE DRYER 4.5 (LABCONCO, Kansas City,  
15 MO). The resulting ammonium bicarbonate salts of Met-rat *ob* protein were shown to be soluble to at least 10mg/mL in water. Both preparations of the ammonium bicarbonate salt form of *ob* protein were tested and shown to be active *in vivo* as described in Example 23.

20      **Example 21 -- *Ob* Proteins Formulated as a Clear, Stable Buffered Solution**

Monomeric or dimeric *ob* proteins may be prepared in a stable liquid formulation using BisTris propane, with or without a detergent (such as Tween 80), and optionally containing a preservative (such as phenol). In this experiment, lyophilized monomeric *ob* protein prepared as described in Example 19 was  
25 dissolved in 0.1% TFA in water to a final concentration of 2.5mg/mL. The pH of the resulting solution was adjusted to 8.4 by the careful addition of 100mM Bis-Tris

propane. The resulting protein solution was transferred into SpectraPor 3500MW cutoff dialysis tubing and fully dialyzed against 20mM Bis-Tris propane pH 8.4 to yield a clear solution. 2 mg/mL solutions of monomeric ob protein in 20 mM BisTris propane (pH 8.4), with and without 0.02% Tween 80 with and without 0.1 M sodium chloride, have been shown to be stable for at least 30 days at room temperature.

**Example 22 – High Yield Production of Met-rat *ob* Protein Monomer and Dimer**

This further set of experiments exemplifies the efficient production of *ob* proteins using the above-described techniques. A seed culture of pET27OPTII from Example 15 was inoculated from a frozen glycerol stock and grown overnight in 150mL of 20g/L LB media (Gibco BRL, Gaithersburg, MD) containing 50mg/L kanamycin (Fisher Biotech, Fair Lawn, NJ) at 37°C to a final OD<sub>600nm</sub> of 3.87. Six 2L Fernbach flasks containing 1L each of sterile filtered 25g/L LB media containing 50mg/L kanamycin were inoculated with 20mL of the of the overnight seed culture, grown at 36°C for 2 hours to an OD<sub>600nm</sub> of 0.875) and production of Met-rat *ob* protein was induced by the addition of IPTG (isopropyl, thio β-G-galactosidase; Fisher Biotech, Fair Lawn, NJ) to a final concentration of 1mM. After induction, the cells were incubated for 4.5h at 37°C and harvested by centrifugation in a JA10 rotor for 15 min. at 5000rpm using a model J2-21 centrifuge (Beckman Instruments Inc. Fullerton, CA). The 19.2g cell pellet was flash frozen and stored at -20°C. The cells were resuspended in 100mM potassium phosphate, pH 6.5 and lysed with two passages through a model 110Y microfluidizer (Microfluidics Corp., Newton, MA) in 500mL of 100mM potassium phosphate buffer, pH 6.5. The inclusion bodies

containing Met-rat *ob* were recovered by centrifugation at 6000xg for 25minutes in an RC-3B centrifuge (Dupont Co., Willmington, DE). The inclusion bodies were resuspended in phosphate buffer and recovered by centrifugation to yield 4.35g of Met-rat *ob* protein-containing inclusion bodies which contained 30% by weight Met-rat *ob* protein.

The inclusion body pellet was dissolved in 725mL of 50mM ammonium bicarbonate buffer containing 8M urea and dialyzed against 20L of 17mM Bis-Tris propane overnight at 4°C in 3500 MW cut-off Spectra-Por dialysis tubing. Prior to dialysis, the inclusion body solution contained 1.2g of monomeric met-rat *ob* protein and 0.07g of dimeric met-rat *ob* protein. After dialysis, the solution contained 0.9g of monomeric Met-rat *ob* protein and 0.27g of dimeric Met-rat *ob* protein. The dialyzed solution was filtered through a Sartobran 300 capsule filter (Sartorius Corp., Tustin, CA) and loaded onto a 29mL Whatman DE-52 (Whatman Inc., Clifton, NJ) anion exchange column at a flow rate of 10mL/min. The column was washed with 17mM BisTris-propane containing 0.02%v/v Tween-80 at pH 8.5 and eluted using a 30min. gradient to 0.5M sodium chloride in the same buffer. Protein elution was monitored by absorbance at 280nm and verified by SDS-PAGE and RP-HPLC analysis of fractions collected during the salt gradient. Appropriate fractions were pooled, acidified to 0.3% TFA and further purified by RP-HPLC on a Vydac 218TP1022 column (Vydac, Hesperia, CA) using a 20 minute gradient from 0.1%TFA in 45% acetonitrile to 0.1% TFA in 65% acetonitrile. Fractions were collected during the acetonitrile gradient and Met-rat *ob* protein elution was monitored using absorbance at 214nm. The monomeric and dimeric forms of Met-rat *ob* protein were pooled separately and concentrated for 1h on a Speedvac Plus



SC110A (Savant, Farmingdale, NY) to remove acetonitrile, and then brought to pH 7 by the careful addition of 0.5M ammonium bicarbonate. Each of the neutralized protein solutions (194mL of monomer and 108mL of dimer) were dialyzed against 10L of 20mM ammonium bicarbonate buffer (pH 7.8 when freshly dissolved) with 5 changes of the dialysis buffer using SpectraPor 3500MW cutoff membranes. The dialyzed protein solutions were filtered, flash frozen and lyophilized on a FREEZE DRYER 4.5 (LABCONCO, Kansas City, MO) to yield 428mg of monomeric Met-rat *ob* protein and 74mg of dimeric Met-rat *ob* protein. Both proteins appeared  $\geq 95\%$  pure by analytical HPLC and SDS-PAGE. The overall yield of the process was 40%.

#### Example 23 -- *In vivo* Testing of Bioactivity of *ob* Proteins

The *ob* protein to be tested in these experiments was dissolved at 2mg/mL in water at room temperature with vortexing. If necessary the solution was allowed to stand for 30 min. and vortexed again. The solution was then diluted to 1mg/mL by the addition of 34mM BisTris-propane containing 0.02% Tween-80, pH 8.5 and stored at room temperature prior to use.

10 - 17 week old *ob/ob* mice (C57BL/6J-*ob/ob*) were utilized for the study. Mice were obtained from Jackson Laboratories, Bar Harbor, ME and housed in groups of 10 animals in the vivarium with 12:12 light:dark cycle with room temperature of  $23 \pm 1$  °C. One week prior to the start of experiments, the animals were divided in to groups of either 4 or 6 animals per group and housed 2 animals per cage. Daily food intake and body weight data were collected and utilized as baseline.

10-14 week old *NIH/Sw* mice were utilized for the study. Mice were obtained from Harlan Laboratories, Madison, WI and housed in groups of 10 animals in the vivarium with 12:12 light:dark cycle with room temperature of  $23 \pm 1$  °C. One week prior to the start of experiments, the animals were divided in to  
5 groups of either 4 or 6 animals per group and housed 2 animals per cage. Daily food intake and body weight data were collected and utilized as baseline.

All animals received subcutaneous injection of either vehicle or test material twice daily for five days. Morning injections were given between 6 and 7 am and evening injections were given between 5 and 6 PM. Daily food intake and body  
10 weight data was collected for all animals. Deviations from baseline food intake and body weight were calculated on a daily basis. A dose response curve of % decrease in body weight and % decrease in food intake, as observed after five days of treatment was plotted using Graphpad Prizm, San Diego, CA.

The compounds of the invention had the following effects on food intake.  
15 Rat *ob* protein, prepared as in Example 14, dose dependently reduced food intake in the *ob/ob* mice. See Figure 2. The  $ED_{50}$  for rat *ob* protein, for reduction in food intake after 5 days of treatment was calculated to be  $0.04 \text{ mg/kg} \pm 0.06 \text{ log units}$  given twice a day.

Met-rat *ob* monomer, prepared as in Example 20, was tested at two doses  
20 (0.05 and 0.5 mg/kg, twice a day) and showed effect on reduction in food intake, after five days of treatment, similar to rat *ob* protein. See Figure 3.

Met-rat *ob* dimer, prepared as in Example 22, was tested at three doses (0.03, 0.3 and 3.0 mg/kg, twice a day) and showed a dose dependent reduction in food intake. See Figure 4. The  $ED_{50}$  for Met-rat *ob* dimer, for reduction in food intake,

after 5 days of treatment, was calculated to be 0.41 mg/kg  $\pm$  0.20 log units given twice a day.

FLAG-rat *ob* monomer, prepared as in Example 11, was tested at two doses (0.10 and 1.0 mg/kg, twice a day) and showed effect on reduction in food intake  
5 after five days of treatment, similar to rat *ob* protein. See Figure 5.

FLAG-rat *ob* dimer, prepared as in Example 11, was tested at two doses (0.1 and 1.0 mg/kg, twice a day) and showed effect on reduction in food intake after five days of treatment, similar to Met-rat *ob* dimer. See Figure 6.

Rat *ob* protein, prepared as in Example 14, also dose dependently reduced  
10 food intake in the *NIH/Sw* mice. See Figure 7. The ED<sub>50</sub> for rat *ob* protein, for reduction in food intake after 5 days of treatment was calculated to be 0.01 mg/kg  $\pm$  0.87 log units given twice a day in *NIH/Sw* mice.

The compounds of the invention had the following effects on body weight.  
Rat *ob* protein, prepared as in Example 14, dose dependently reduced body weight in  
15 the *ob/ob* mice. See Figure 8. The ED<sub>50</sub> for rat *ob* protein, for reduction in body weight after 5 days of treatment was calculated to be 0.09 mg/kg  $\pm$  0.14 log units given twice a day.

Met-rat *ob* monomer, prepared as in Example 20, was tested at two doses (0.05 and 0.5 mg/kg, twice a day) and showed effect on reduction in body weight,  
20 after five days of treatment, similar to rat *ob* protein. See Figure 9.

Met-rat *ob* dimer, prepared as in Example 22, was tested at three doses (0.03, 0.3 and 3.0 mg/kg, twice a day) and showed a dose dependent reduction in body weight. See Figure 10. The ED<sub>50</sub> for Met-rat *ob* dimer, for reduction in body

weight, after 5 days of treatment, was calculated to be 1.01 mg/kg  $\pm$  0.21 log units given twice a day.

FLAG-rat *ob* monomer, prepared as in Example 11, was tested at two doses (0.10 and 1.0 mg/kg, twice a day) and showed effect on reduction in body weight after five days of treatment, similar to rat *ob* protein. See Figure 11.

FLAG-rat *ob* dimer, prepared as in Example 11, was tested at two doses (0.1 and 1.0 mg/kg, twice a day) and showed effect on reduction in body weight after five days of treatment, similar to Met-rat *ob* dimer. See Figure 12.

Rat *ob* protein, prepared as in Example 14, also dose dependently reduced body weight in the *NIH/Sw* mice. See Figure 13. The ED<sub>50</sub> for rat *ob* protein, for reduction in body weight after 5 days of treatment was calculated to be 0.06 mg/kg  $\pm$  0.15 log units given twice a day in *NIH/Sw* mice.

#### Example 24 – *E. coli* Expression of Met-human *ob* Protein

In order to prepare a vector construct for high level production of Met-human *ob* protein, the coding region for mature human *ob*, Val<sup>22</sup> to Cys<sup>146</sup>, was PCR amplified from a cloning vector using the reagents from the Perkin Elmer PCR kit (Perkin Elmer, Foster City, CA). The upstream primer, 5'- TATACAT ATG GTT CCG ATC CAG AAA GTC CAA GAT GAC, incorporated an *Nde*I site (underlined) onto the 5' end of the PCR fragment to facilitate cloning. The *Nde*I site also has within it the initiating methionine codon, ATG. The included codons for Val<sup>22</sup> (GTT), Pro<sup>23</sup> (CCG), and Gln<sup>23</sup> (CAG) of the human *ob* protein have been changed from the original DNA sequence to preferred codons for *E. coli* expression. The downstream primer used for PCR was a vector (pET27b+, Novagen Inc., Madison, WI) specific, T7 terminator primer. PCR amplification was performed in a

100 µl volume containing 50ng of template DNA, 0.5µM each primer, 2.5 units of AmpliTaq DNA® Polymerase, 10mM Tris-HCl, pH 8.3, 50mM KCl, 0.2mM each dNTP, and 2.0mM MgCl<sub>2</sub>. The reaction was incubated for 2 cycles at 95°C/1 min, 47°C/1 min, and 72°C/1 min followed by 30 cycles at 95°C/30 sec, 65°C/ 30 sec, and 72°C/ 30 sec followed by a final incubation of 10 min at 72°C. Analytical agarose gel electrophoresis confirmed a single band of the expected size, approximately 650bp. The remaining PCR reaction material was ethanol precipitated, resuspended in water and ligated into the holding vector, pCRII (TA Cloning Kit, Invitrogen Corp., San Diego, CA) following kit protocol. Positive clones were selected and characterized to confirm the presence of the inserted PCR fragment. The Met-human *ob* coding region was isolated from the pCR vector by digesting the vector DNA with the restriction enzymes *Nde*I and *Xho*I. The insert fragment, approximately 540bp, was isolated from an agarose gel and purified using a silica membrane system (SpinBind® DNA Recovery System; FMC BioProducts, Rockland, ME) following the manufacturer's protocol. The fragment was ligated into the pET27b+ vector also cut with *Nde*I and *Xho*I. The resulting vector, pET27HOPT#1, was sequenced to confirm proper construction of the coding region.

#### **Example 25 – High Yield Production of Met-human *ob* Protein Monomer and Dimer**

20

This further set of experiments exemplifies the efficient production of Met-human *ob* proteins using the above-described techniques. A seed culture of pET27HOPT#1 from Example 24 was inoculated from a previously prepared plate and grown overnight in 150mL of 25g/L LB media (Gibco BRL, Gaithersburg, MD) containing 50mg/L kanamycin (Fisher Biotech, Fair Lawn, NJ) at 37°C to a final

25

OD<sub>600nm</sub> of 3.33. Six 2L Fernbach flasks containing 1L each of sterile filtered 25g/L LB media containing 50mg/L kanamycin were inoculated with 20mL of the of the overnight seed culture, grown at 36°C-37°C for 2 hours to an OD<sub>600nm</sub> of 0.801) and production of Met-human *ob* protein was induced by the addition of IPTG (isopropyl, thio  $\beta$ -G-galactosidase; Fisher Biotech, Fair Lawn, NJ) to a final concentration of 1mM. After induction, the cells were incubated for 4h at 37°C and harvested by centrifugation in a JA10 rotor for 15 min. at 5000rpm using a model J2-21 centrifuge (Beckman Instruments Inc. Fullerton, CA). The 15.9g cell pellet was resuspended in 100mM potassium phosphate, pH 6.5 and lysed with two passages through a model 110Y microfluidizer (Microfluidics Corp., Newton, MA) in 500mL of 100mM potassium phosphate buffer, pH 6.5. The inclusion bodies containing Met-human *ob* were recovered by centrifugation at 6000xg for 30minutes in an RC-3B centrifuge (Dupont Co., Willmington, DE). The inclusion bodies were resuspended in phosphate buffer and recovered by centrifugation to yield 3.9g of Met-human *ob* protein-containing inclusion bodies.

The inclusion body pellet was dissolved in 650mL of 50mM ammonium bicarbonate buffer containing 8M urea and dialyzed against 20L of 17mM Bis-Tris propane overnight at 4°C in 3500 MW cut-off Spectra-Por dialysis tubing. After dialysis, the solution contained 0.9g of monomeric Met-human *ob* protein and 0.27g of dimeric Met-human *ob* protein. The dialyzed solution was filtered through a Sartobran 300 capsule filter (Sartorius Corp., Tustin, CA) and loaded onto a 16mL Whatman DE-52 (Whatman Inc., Clifton, NJ) anion exchange column at a flow rate of 10mL/min. The column was washed with 17mM BisTris-propane containing 0.02%v/v Tween-80 at pH 8.5 and eluted using a 30minutes gradient to 0.5M

sodium chloride in the same buffer. Protein elution was monitored by absorbance at 280nm and determined by RP-HPLC analysis of fractions collected during the salt gradient. to yield 600 mg of monomeric Met-human *ob* protein and 42mg of dimeric Met-human *ob* protein.

5           **Example 26 -- Periplasmic expression of FLAG-human *ob* protein**

In order to prepare a vector construct for periplasmic expression of FLAG-human *ob* protein, the coding region for human *ob* was inserted into a vector construct as described in Example 4. A seed culture of vector pFLAGATSHOB#5 in W3110 cells was inoculated from a previously prepared plate and grown  
10 overnight at 37°C in 150mL of 20g/L LB media (Gibco BRL, Gaithersburg, MD) containing 50mg/L ampicillin to a final OD<sub>600nm</sub> of 3.46. Six 2L Fernbach flasks containing 1L each of sterile filtered 25g/L LB media (Gibco BRL, Gaithersburg, MD) containing 50mg/L ampicillin were inoculated with 20mL of the overnight seed culture. The six flasks were then grown at 27°C for 3.5 hours to an OD<sub>600nm</sub> of  
15 0.401 in the presence of 5g/L glucose and production of FLAG-human *ob* protein was induced by the addition of IPTG (isopropyl, thio β-G-galactosidase; Fisher Biotech, Fair Lawn NJ) to a final concentration of 0.5mM. After induction, the cells were incubated for 3h at 28°C to a final OD<sub>600nm</sub> of 1.94 and harvested by centrifugation in a JA10 rotor for 15 min. at 5000rpm using a model J2-21  
20 centrifuge (Beckman Instruments Inc., Fullerton, CA). The 18.0g cell pellet obtained in this manner was then washed three times by resuspending the pellet to 25g/L with 10mM Tris buffer, pH 8.0 and centrifuging the cells at 5000rpm for 10 minutes in a Beckman JA-10 rotor. The washed cell pellet was then resuspended to 25g/L with 30mM Tris buffer containing 0.5M sucrose and 1mM EDTA at pH 8.0

and incubated at room temperature for 10 minutes to allow the cells to equilibrate. The cells were then removed from the sucrose-containing buffer by centrifugation, as above, and shocked by resuspension to 40g/L with ice-cold water containing 1mM Pefflabloc SC (Centerchem, Stamford, CT). The periplasmic extract obtained  
5 in this manner was clarified by centrifugation at 8500rpm for 10 minutes in a JA-10 rotor using a model J2-21 centrifuge (Beckman Instruments Inc., Fullerton, CA) and stored at 4°C until further purification. The yield of FLAG-human *ob* protein from this protocol was 58.5mg by HPLC analysis

Tween-80 was added to the periplasmic extracts to a final concentration of  
10 0.02%w/v and the pH of the resulting solution was adjusted to 8.5 by the careful addition of 100mM BisTris-propane free base in water. The protein solution was then clarified by filtration through an 0.45µm Sartobran filter (Sartorius Corp., Tustin, CA). The clarified solution was loaded onto DE-52 resin (Whatman Inc., Clifton, NJ) packed into an XK16 column (Pharmacia Biotech, Piscataway, NJ) to a  
15 bed height of 4.5cm at a flow rate of 4mL/minute. The column was washed with 17mM BisTris-propane containing 0.02% Tween-80 at pH 8.5 and the FLAG-human *ob* protein was eluted using a linear gradient to 0.5M NaCl in 17mM BisTris-propane buffer containing 0.02% Tween-80 at pH 8.5. Protein elution was monitored using absorbance detection at 280nm and verified by SDS-PAGE and RP-  
20 HPLC analysis of fractions collected during the elution gradient. Appropriate fractions were pooled, brought to pH 2.5 by the addition of TFA to 0.3% v/v and loaded onto a VYDAC 218TP1022 HPLC column (Vydac, Hesperia, CA) using a flow rate of 15mL/min. The FLAG-human *ob* protein was eluted from the column using a linear gradient from 0.1%TFA in 45% acetonitrile to 0.1% TFA in 60%



acetonitrile over 15 minutes. The elution of FLAG-human *ob* protein was detected by absorbance at 214nm and confirmed by SDS-PAGE. Monomeric FLAG-human *ob* protein was concentrated by evaporation using a Speedvac Plus SC110A (Savant, Farmingdale, NY), flash frozen and lyophilized as TFA salts using a FREEZE  
5 DRYER 4.5 (LABCONCO, Kansas City, MO). The proteins obtained in this manner were  $\geq 95\%$  pure by SDS-PAGE analysis.

Various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing  
10 description and fall within the scope of the following claims.

**WE CLAIM:**

1. A method for the production of an *ob* dimer which comprises the steps of
  - (a) isolating a vertebrate cDNA library,
  - (b) ligating said cDNA library into a cloning vector,
  - 5 (c) introducing said cloning vector containing said cDNA library into  
a first host cell,
  - (d) contacting the cDNA molecules of said first host cell with a  
solution containing a suitable *ob* gene hybridization probe,
  - (e) detecting a cDNA molecule which hybridizes to said probe,
  - 10 (f) isolating said cDNA molecule,
  - (g) ligating the nucleic acid sequence of said cDNA molecule which  
encodes an *ob* protein into an expression vector,
  - (h) transforming a second host cell with said expression vector  
containing said nucleic acid sequence of said cDNA molecule which encodes  
15 said *ob* protein,
  - (i) culturing the transformed second host cell under conditions that  
favor the production of said *ob* protein as a dimer, and
  - (j) isolating said *ob* protein expressed by said second host cell.
2. The method of claim 1 wherein said vertebrate cDNA library comprises a  
20 vertebrate adipose tissue cDNA library.
3. The method of claim 2 wherein said vertebrate adipose tissue cDNA  
library comprises a human adipose tissue cDNA library.
4. A method of producing a *ob* dimer which comprises the steps of

(a) culturing a transformed host cell containing a DNA sequence encoding a vertebrate *ob* protein under conditions that favor the production of said vertebrate *ob* protein as a dimer, and

(b) isolating said *ob* dimer expressed by said transformed host cell.

5        5. The method of claim 4 wherein said vertebrate *ob* protein is a human *ob* protein.

6. A method of producing a *ob* dimer which comprises the steps of

(a) culturing a transformed host cell containing a DNA sequence encoding a vertebrate *ob* protein under conditions that favor the production of

10       said vertebrate *ob* protein as a monomer,

(b) isolating said *ob* protein expressed by said transformed host cell,

and

(c) dimerizing said *ob* protein.

7. The method of claim 6 wherein said vertebrate *ob* protein is a human *ob*  
15       protein.

8. A method for the production of an *ob* dimer which comprises the steps of

(a) isolating a preparation of total RNA from a vertebrate tissue,

(b) converting said total RNA to cDNA,

(c) amplifying a cDNA sequence from said cDNA using  
20       oligonucleotide primers suitable for annealing to a target *ob* protein gene sequence,

(d) detecting a cDNA molecule using oligonucleotides suitable for hybridization to said target *ob* protein gene sequence,

- (e) isolating said cDNA molecule,
- (f) ligating the nucleic acid sequence of said cDNA molecule which encodes an *ob* protein into an expression vector,
- (g) transforming a host cell with said expression vector containing
- 5 said nucleic acid sequence of said cDNA molecule which encodes said *ob* protein,
- (h) culturing the transformed host cell under conditions that favor the production of said *ob* protein as a dimer, and
- (i) isolating said *ob* protein expressed by said host cell.

9. The method of claim 8 wherein said vertebrate tissue is a vertebrate  
10 adipose tissue.

10. The method of claim 9 wherein said vertebrate adipose tissue is human adipose tissue.

11. A method for the production of an *ob* dimer fusion protein or *ob* monomer fusion protein which comprises the steps of

- 15 (a) isolating a vertebrate cDNA library,
- (b) ligating said cDNA library into a cloning vector,
- (c) introducing said cloning vector containing said cDNA library into
- a first host cell,
- (d) contacting the cDNA molecules of said first host cell with a
- 20 solution containing a suitable *ob* gene hybridization probe,
- (e) detecting a cDNA molecule which hybridizes to said probe,
- (f) isolating said cDNA molecule,

(g) ligating the nucleic acid sequence of said cDNA molecule which encodes an *ob* protein to a second DNA sequence to create a fusion DNA sequence,

(h) ligating said fusion DNA sequence into an expression vector,

5 (i) transforming a second host cell with said expression vector containing said fusion DNA sequence,

(j) culturing the transformed second host cell under conditions that favor the production of said *ob* fusion protein as a dimer or monomer, and

(k) isolating said *ob* protein expressed by said second host cell.

10 12. The method of claim 11 wherein said vertebrate cDNA library comprises a vertebrate adipose tissue cDNA library.

13. The method of claim 12 wherein said vertebrate adipose tissue cDNA library comprises a human adipose tissue cDNA library.

14. A method of producing an *ob* dimer fusion protein or *ob* monomer  
15 fusion protein which comprises the steps of

(a) culturing a transformed host cell containing a DNA sequence encoding a vertebrate *ob* protein coupled to a marker DNA sequence under conditions that favor the production of said vertebrate *ob* fusion protein as a dimer or monomer, and

20 (b) isolating said *ob* dimer fusion protein or *ob* monomer fusion protein expressed by said transformed host cell.

15. The method of claim 14 wherein said vertebrate *ob* protein is a human *ob* protein.

16. A method of producing an *ob* dimer fusion protein which comprises the steps of

(a) culturing a transformed host cell containing a DNA sequence encoding a vertebrate *ob* protein coupled to a marker DNA sequence under conditions that favor the production of said vertebrate *ob* fusion protein as a monomer,

(b) isolating said *ob* fusion protein expressed by said transformed host cell, and

(c) dimerizing said *ob* fusion protein.

17. The method of claim 16 wherein said vertebrate *ob* protein is a human *ob* protein.

18. A method for the production of an *ob* dimer fusion protein or *ob* monomer fusion protein which comprises the steps of

(a) isolating a preparation of total RNA from a vertebrate tissue,

(b) converting said total RNA to cDNA,

(c) amplifying a cDNA sequence from said cDNA using oligonucleotide primers suitable for annealing to a target *ob* protein gene sequence,

(d) detecting a cDNA molecule using oligonucleotides suitable for hybridization to said target *ob* protein gene sequence,

(e) isolating said cDNA molecule,

(f) ligating the nucleic acid sequence of said cDNA molecule which encodes an *ob* fusion protein to a second DNA sequence to create a fusion DNA sequence encoding an *ob* fusion protein

- (g) ligating said fusion DNA sequence into an expression vector,
- (h) transforming a host cell with said expression vector containing  
said fusion DNA sequence,
- (i) culturing the transformed host cell under conditions that favor the  
5 production of said *ob* fusion protein as a dimer or monomer, and
- (j) isolating said *ob* fusion protein expressed by said host cell.
19. The method of claim 18 wherein said vertebrate tissue is a vertebrate  
adipose tissue.
20. The method of claim 19 wherein said vertebrate adipose tissue is human  
10 adipose tissue.
21. A composition comprising an isolated *ob* dimer.
22. The composition of claim 21 wherein said *ob* dimer is a human *ob*  
dimer.
23. The composition of claim 21 wherein said *ob* dimer is a rat *ob* dimer.
- 15 24. The composition of claim 21 wherein said *ob* dimer is a mouse *ob* dimer.
25. A composition comprising an isolated *ob* dimer fusion protein.
26. The composition of claim 25 wherein said *ob* dimer fusion protein is a  
human *ob* dimer fusion protein.
27. The composition of claim 25 wherein said *ob* dimer fusion protein is a  
20 rat *ob* dimer fusion protein.
28. The composition of claim 25 wherein said *ob* dimer fusion protein is a  
mouse *ob* dimer fusion protein.
29. A pharmaceutical composition for use in the treatment of conditions or  
disorders that would benefit from reduced food intake or increased energy

expenditure, which comprises a therapeutically effective amount of an *ob* dimer in association with a pharmaceutically acceptable carrier.

30. The pharmaceutical composition of claim 29 wherein said *ob* dimer is a human *ob* dimer.

5        31. A pharmaceutical composition for use in the treatment of conditions or disorders that would benefit from reduced food intake or increased energy expenditure, which comprises a therapeutically effective amount of an *ob* dimer fusion protein in association with a pharmaceutically acceptable carrier.

10       32. The pharmaceutical composition of claim 31 wherein said *ob* dimer is a human *ob* dimer.

33. A method for the treatment of conditions or disorders in a subject that would benefit from reduced food intake or increased energy expenditure, which comprises administering to said subject an amount of an *ob* dimer effective to suppress appetite in said subject.

15       34. The method of claim 33 wherein said condition or disorder is obesity.

35. The method of claim 33 wherein said condition or disorder is diabetes.

36. The method of claim 35 wherein said diabetes condition or disorder is Type 2 diabetes.

20       37. The method of any of claims 33-35 or 36 wherein said *ob* dimer is a human *ob* dimer.

38. A method for the treatment of conditions or disorders in a subject that would benefit from reduced food intake or increased energy expenditure, which comprises administering to said subject an amount of an *ob* dimer fusion protein effective to reduce appetite in said subject.



39. The method of claim 38 wherein said condition or disorder is obesity.

40. The method of claim 38 wherein said condition or disorder is diabetes.

41. The method of claim 40 wherein said diabetes condition or disorder is Type 2 diabetes.

5        42. The method of any of claims 38-40 or 41 wherein said *ob* dimer fusion protein is a human *ob* dimer fusion protein.

43. A monoclonal antibody which binds to an *ob* dimer, wherein said monoclonal antibody recognizes said *ob* dimer with greater specificity than the *ob* monomer of which said *ob* dimer is comprised.

10       44. The monoclonal antibody of claim 43 wherein said *ob* dimer is a human *ob* dimer.

45. The monoclonal antibody of claim 43 wherein said *ob* dimer is selected from the group consisting of a rat *ob* dimer and a mouse *ob* dimer.

46. A monoclonal antibody which binds to an *ob* dimer fusion protein,  
15 wherein said monoclonal antibody recognizes said *ob* dimer fusion protein with greater specificity than the *ob* monomer fusion protein of which said *ob* dimer fusion protein is comprised.

47. The monoclonal antibody of claim 46 wherein said *ob* dimer fusion protein is a human *ob* dimer fusion protein.

20       48. The monoclonal antibody of claim 46 wherein said *ob* dimer fusion protein is selected from the group consisting of a rat *ob* dimer fusion protein and a mouse *ob* dimer fusion protein.

49. An assay using a monoclonal antibody for detecting the presence or amount of an *ob* dimer comprising the steps of:

(a) contacting said *ob* dimer with said monoclonal antibody, wherein said monoclonal antibody binds to said *ob* dimer with greater specificity than the *ob* monomer of which said *ob* dimer is comprised, and

(b) determining the presence or amount of said *ob* dimer.

5           50. The assay of claim 49 wherein a second monoclonal antibody to said *ob* dimer or a polyclonal antibody to said *ob* dimer is used in said assay.

51. The assay of claim 49 wherein a second monoclonal antibody to the *ob* monomer of which said *ob* dimer is comprised or a polyclonal antibody to the *ob* monomer of which said *ob* dimer is comprised is used in said assay.

10           52. The assay of claim 50 or 51 wherein said second monoclonal antibody is detectably labeled.

53. The assay of claim 49 wherein said assay is a competitive assay.

54. The assay of claim 49 wherein said assay is a sandwich assay.

15           55. An assay using a monoclonal antibody for detecting the presence or amount of an *ob* dimer fusion protein comprising the steps of:

(a) contacting said *ob* dimer fusion protein with said monoclonal antibody, wherein said monoclonal antibody binds to said *ob* dimer fusion protein with greater specificity than the *ob* monomer fusion protein of which said *ob* dimer fusion protein is comprised, and

20           (b) determining the presence or amount of said *ob* dimer fusion protein.

56. The assay of claim 55 wherein a second monoclonal antibody to said *ob* dimer fusion protein or a polyclonal antibody to said *ob* dimer fusion protein is used in said assay.

57. The assay of claim 55 wherein a second monoclonal antibody to the *ob* monomer fusion protein of which said *ob* dimer fusion protein is comprised or a polyclonal antibody to the *ob* monomer fusion protein of which said *ob* dimer fusion protein is comprised is used in said assay.

5 58. The assay of claim 56 or 57 wherein said first or said second monoclonal antibody is detectably labeled.

59. The assay of claim 55 wherein said assay is a competitive assay.

60. The assay of claim 55 wherein said assay is a sandwich assay.

61. An assay for determining the presence or amount of an *ob* dimer or *ob* dimer fusion protein in a sample suspected of containing an *ob* dimer or *ob* dimer fusion protein, comprising the steps of

(a) contacting said sample suspected of containing an *ob* dimer or *ob* dimer fusion protein with a monoclonal antibody according to any of claims 43, 44, 46 or 47;

15 (b) contacting positive and/or negative control samples with a monoclonal antibody according to any of claims 43, 44, 46 or 47; and

(c) determining the presence or amount of said *ob* dimer or *ob* dimer fusion protein.

62. The assay according to claim 61 wherein said assay is a competitive  
20 assay.

63. The assay according to claim 61 wherein said assay is a sandwich assay.

64. The assay according to claim 61 wherein said monoclonal antibody is detectably labeled and wherein said labeled monoclonal antibody binds to said *ob*

dimer fusion protein and is used to determine the presence or amount of said *ob* dimer fusion protein.

65. An assay for determining the presence or amount of an *ob* dimer or *ob* dimer fusion protein amylin in fluid sample suspected of containing an *ob* dimer or
- 5 *ob* dimer fusion protein, comprising the steps of:

(a) contacting said sample with a measured amount of a first antibody directed to an *ob* dimer or *ob* dimer fusion protein to form a soluble complex of said first antibody and said *ob* dimer or *ob* dimer fusion protein present in said sample, said first antibody being labeled;

- 10 (b) contacting said soluble complex with a second antibody directed to said *ob* dimer or *ob* dimer fusion protein, said second antibody being bound to a solid carrier, to form a ternary complex of said first antibody, said *ob* dimer or *ob* dimer fusion protein and said second antibody bound to said solid carrier;

- 15 (c) separating said solid carrier from said sample and unreacted labeled first antibody;

(d) measuring either the amount of labeled first antibody associated with said solid carrier or the amount of unreacted labeled first antibody; and

- (e) relating the amount of labeled antibody with the amount of
- 20 labeled antibody measured for a control sample prepared in accordance with steps (a)-(d), said control sample being known to be free of said *ob* dimer or *ob* dimer fusion protein, to determine the presence of *ob* dimer or *ob* dimer fusion protein in said fluid sample, or relating the amount of labeled antibody measured with the amount of labeled antibody measured for samples

containing known amounts of *ob* dimer or *ob* dimer fusion protein prepared in accordance with steps (a)-(d) to determine the amount of *ob* dimer or *ob* dimer fusion protein in said fluid sample, said first or second antibody being according to any of claims 43, 44, 46 or 47.

5        66. An assay for determining the presence or amount of *ob* dimer or *ob* dimer fusion protein in a fluid sample suspected of containing said *ob* dimer or *ob* dimer fusion protein comprising the steps of:

(a) contacting said sample with a first antibody directed to said *ob* dimer or *ob* dimer fusion protein, wherein said first antibody is bound to a solid  
10 carrier, to form a complex of said *ob* dimer fusion protein present in said sample and said first antibody;

(b) separating unreacted sample from said complex;

(c) contacting said complex with a measured amount of a second antibody directed to said *ob* dimer or *ob* dimer fusion protein, wherein said  
15 second antibody is labeled;

(d) measuring either the amount of labeled second antibody associated with said complex or the amount of unreacted labeled second antibody; and

(e) relating the amount of labeled antibody with the amount of  
20 labeled antibody measured for a control sample prepared in accordance with steps (a)-(d), said control sample being known to be free of *ob* dimer or *ob* dimer fusion protein, to determine the presence of *ob* dimer or *ob* dimer fusion protein in said fluid sample, or relating the amount of labeled antibody measured with the amount of labeled antibody measured for samples containing known amounts

of *ob* dimer or *ob* dimer fusion protein prepared in accordance with steps (a)-(d) to determine the amount of *ob* dimer or *ob* dimer fusion protein in said fluid sample, said first or second antibody being according to any of claims 43, 44, 46 or 47.

5 67. In an immunometric assay to determine the presence or amount of *ob* dimer or *ob* dimer fusion protein in a sample suspected of containing *ob* dimer or *ob* dimer fusion protein, the improvement comprising employing a monoclonal antibody according to any one of claims 43, 44, 46 or 47.

68. An assay for determining the presence or amount of *ob* dimer or *ob* dimer fusion protein in a sample suspected of containing *ob* dimer or *ob* dimer fusion protein, comprising the steps of:

(a) contacting said sample with a known quantity of added labeled *ob* dimer or *ob* dimer fusion protein;

(b) contacting said sample with a monoclonal antibody according to  
15 any of claims 43, 44, 46 or 47; and

(c) determining the amount of labeled *ob* dimer or *ob* dimer fusion protein bound to said monoclonal antibody or the amount of labeled *ob* dimer or *ob* dimer fusion protein which is not bound to said monoclonal antibody.

69. The assay of any of claims 49-51, 53 or 54 wherein said *ob* dimer is a  
20 human *ob* dimer.

70. The assay of any of claims 55-57, 59 or 60 wherein said *ob* dimer fusion protein is a human *ob* dimer fusion protein

71. A kit comprising a monoclonal antibody which binds to an *ob* dimer, wherein said monoclonal antibody recognizes said *ob* dimer with greater specificity than the *ob* monomer of which said *ob* dimer is comprised. .

72. A kit comprising a monoclonal antibody which binds to an *ob* dimer fusion protein, wherein said monoclonal antibody recognizes said *ob* dimer fusion protein with greater specificity than the *ob* monomer fusion protein of which said *ob* dimer fusion protein is comprised.

73. A kit according to claim 71 or 72 which further comprises suitable control samples.

10 74. A substantially pure *ob* dimer.

75. A pure *ob* dimer.

76. A substantially pure *ob* fusion dimer protein.

77. A pure *ob* fusion dimer protein.

78. The *ob* dimer of claim 74 or 75 which is a human *ob* dimer.

15 79. The *ob* dimer fusion protein of claim 76 or 77 which is a human *ob* dimer fusion protein.

80. A method of treatment which comprises the administration of a therapeutically effective amount of a pharmaceutical composition comprising an *ob* dimer according to claim 74 or 75 to patients in need thereof.

20 81. The method of claim 80 wherein said *ob* dimer is a human *ob* dimer.

82. A method of treatment which comprises the administration of a therapeutically effective amount of a pharmaceutical composition comprising an *ob* dimer fusion protein according to claim 76 or 77 to patients in need thereof.

83. The method of claim 82 wherein said *ob* dimer fusion protein is a human *ob* dimer fusion protein.
84. A composition comprising an *ob* protein in BisTris propane buffer.
85. The composition of claim 84 which further comprises a detergent.
- 5 86. The composition of claim 85 wherein said detergent is a Tween.
87. The composition of any of claims 84, 85 or 86 having a pH of from about 7.5 to about 9.
88. A composition comprising an ammonium bicarbonate salt of an *ob* protein.
- 10 89. The composition of claim 88 wherein said *ob* protein is selected from the group consisting of an *ob* monomer, an *ob* fusion monomer, an *ob* dimer and an *ob* fusion dimer.
90. A method of purifying an *ob* protein which comprises the step of separating said *ob* protein from a sample known to contain said *ob* protein using a cellulose-based anion exchange resin.
- 15 91. The method of claim 90 wherein said cellulose-based anion exchange resin is DE-52 resin.
92. The method of either of claims 90 or 91 which include the use of a BisTris propane running buffer.
- 20 93. The method of claim 90 which further comprises the additional step of purifying the *ob* protein obtained from said cellulose-based anion exchange resin purification step, using reversed phase high pressure liquid chromatography.



94. A composition comprising an isolated *ob* monomer fusion protein.

95. A pharmaceutical composition for use in the treatment of conditions or disorders that would benefit from reduced food intake or increased energy expenditure, which comprises a therapeutically effective amount of an *ob* monomer fusion protein in association with a pharmaceutically acceptable carrier.

96. A method for the treatment of conditions or disorders in a subject that would benefit from reduced food intake or increased energy expenditure, which comprises administering to said subject an amount of an *ob* monomer fusion protein effective to suppress appetite in said subject.

1/13

Fig. 1

1 ATGTGCTGGAGACCCCTGTGCGGTTCTCTGTGGCTTTGGTCCCTATCTGTCTATGTTCAA 60  
M C W R P L C R F L W L W S Y L S Y V Q

61 GCTGTGCCCTATCCACAAAGTCCAGGATGACACCAAAACCCTCATCAAGACCATTTGTCACC 120  
A V P I H K V Q D D T K T L I K T I V T

121 AGGATCAATGACATTTACACACGCGAGTCGGTATCCGCCAGGCAGAGGGTCACCGGTTTG 180  
R I N D I S H T Q S V S A R Q R V T G L

181 GACTTCATTCGCGGGCTTCACCCCATTTCTGAGTTTGTTCCAAGATGGACGACCCCTGGCA 240  
D F I P G L H P I L S L S K M D Q T L A

241 GTCTATCAACAGATCCTCACCAGCTTGCTTCCCAAACGCTGTCAGATAGCTCATGAC 300  
V Y Q Q I L T S L P S Q N V L Q I A H D

301 CTGGAGAACCTGCGAGACCTCCTCCATCTGCTGGCCCTTCTCCAAGAGCTGCTCCCTGCCG 360  
L E N L R D L L H L L A F S K S C S L P

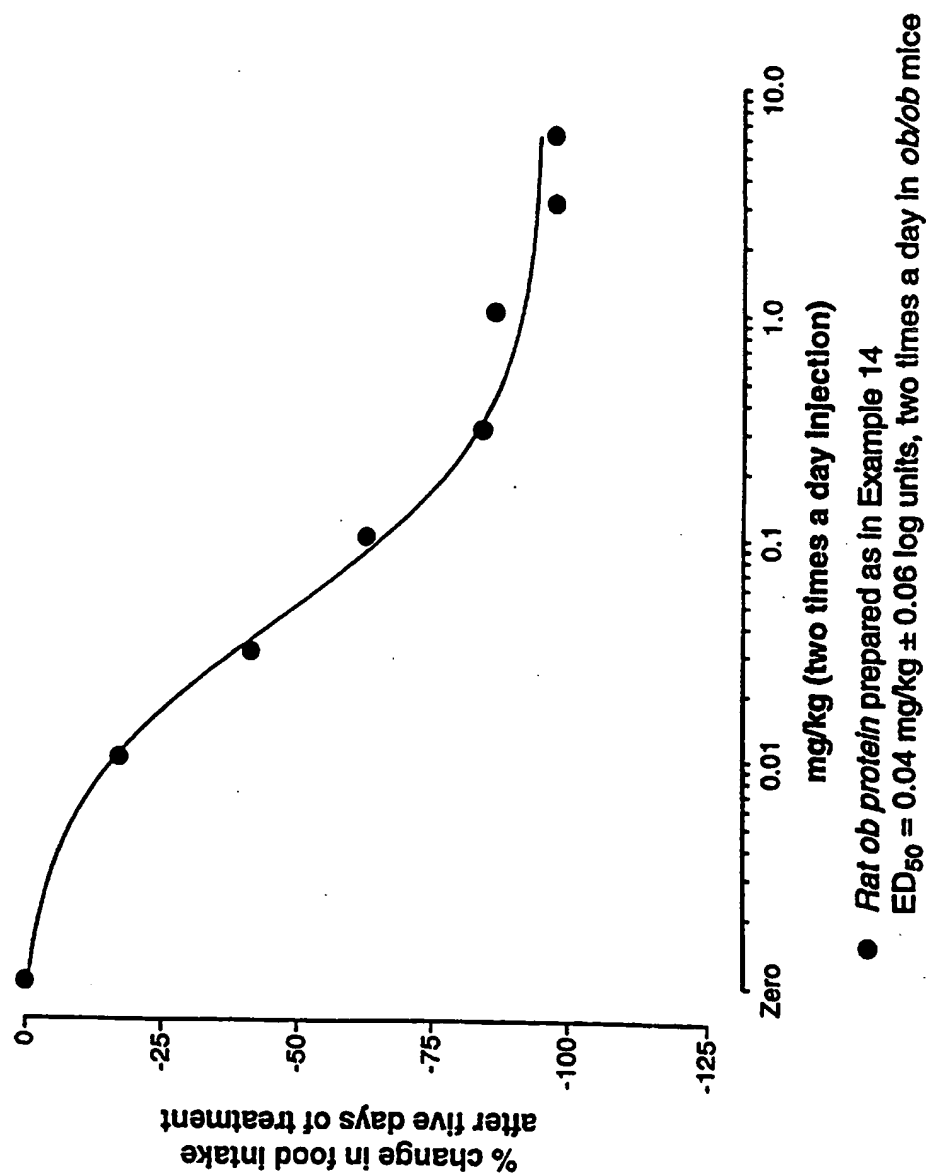
361 CAGACCCGCTGGCCTGCAGAAAGCCAGAGAGCCTGGATGGCGTCTGGAAGCCTCGCTCTAC 420  
Q T R G L Q K P E S L D G V L E A S L Y

421 TCCACAGAGGTGGTCTGTGAGCAGGCTGCAGGGCTCTCTGCAGGACATTTCTTCAACAG 480  
S T E V V A L S R L Q G S L Q D I L Q Q

481 TTGGACCTTAGCCCTGAATGCTGA 504  
L D L S P E C .

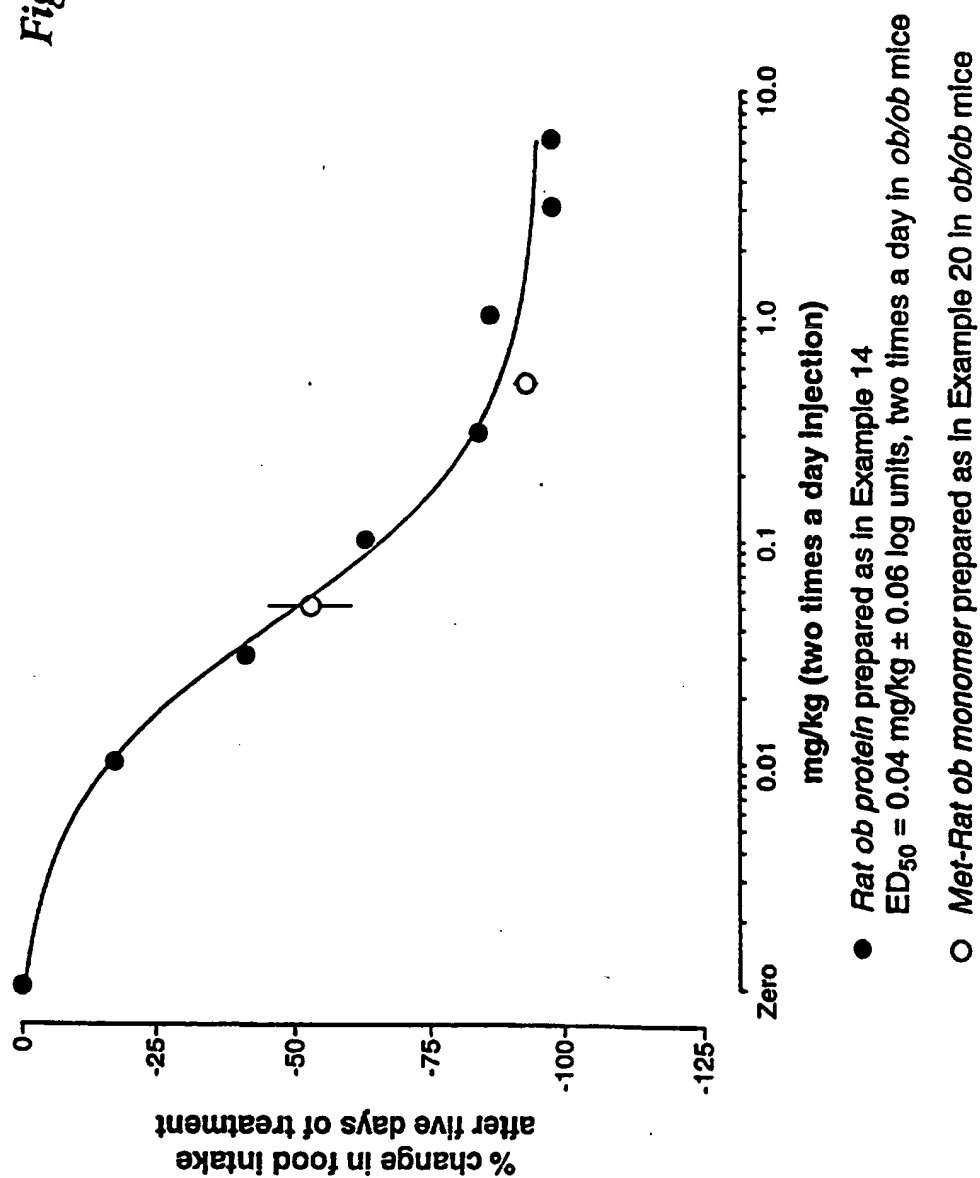
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Fig. 2



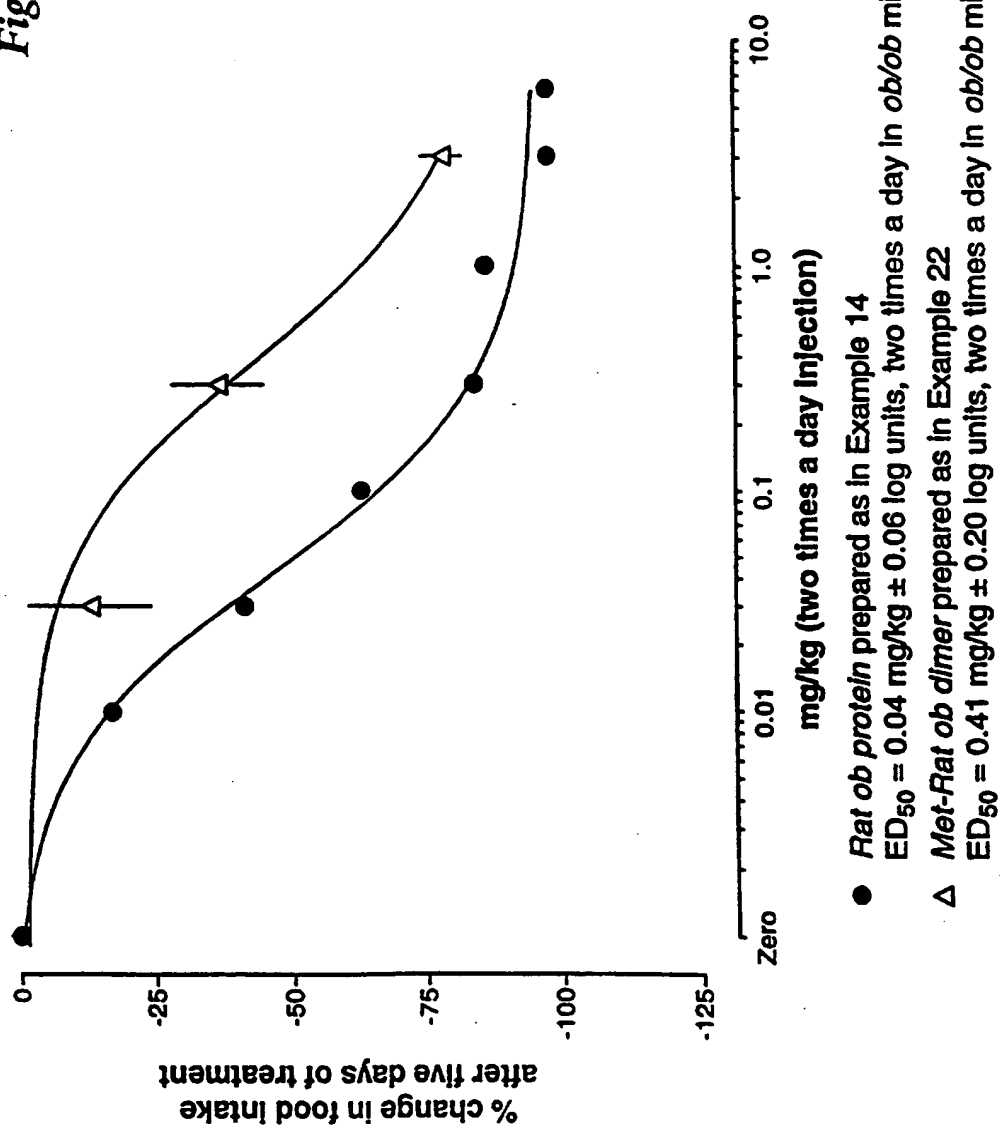
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Fig. 3



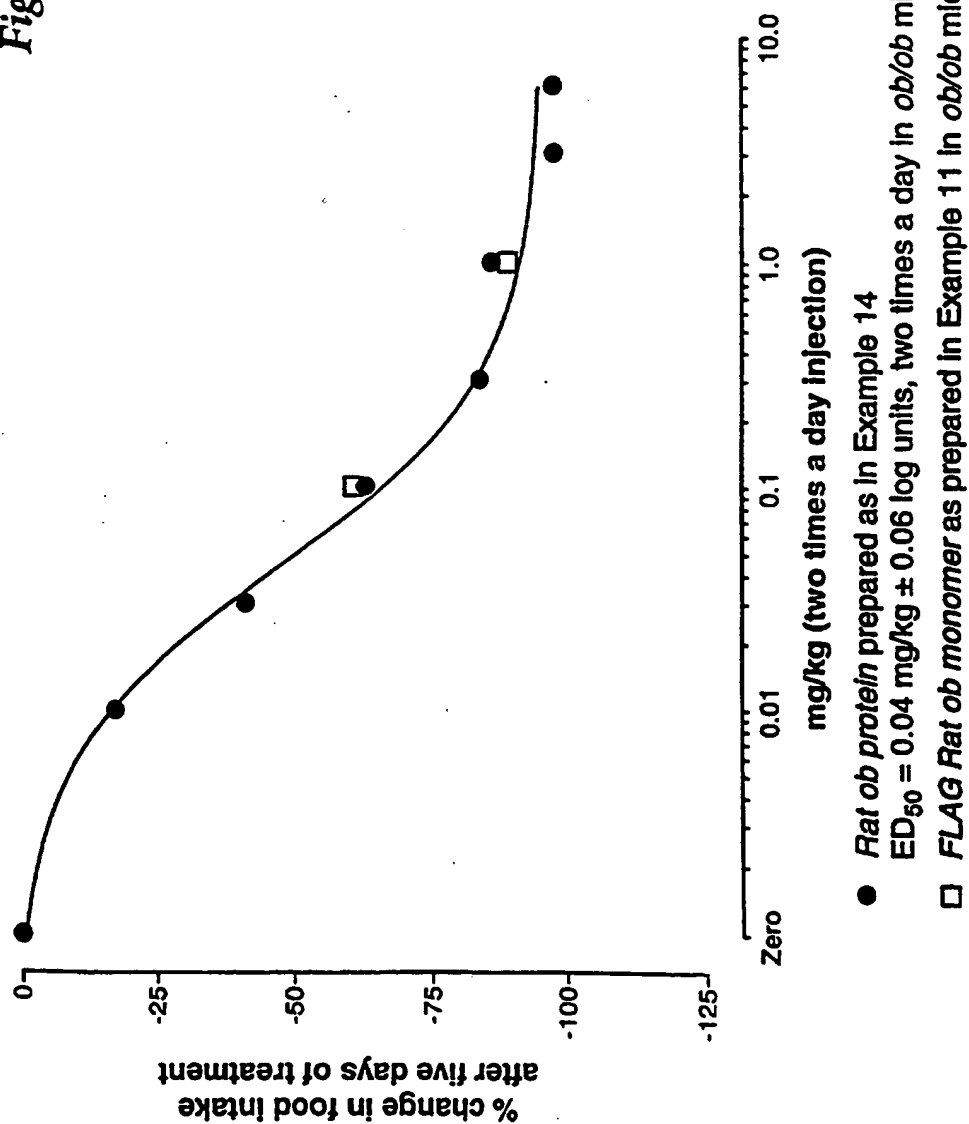
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Fig. 4



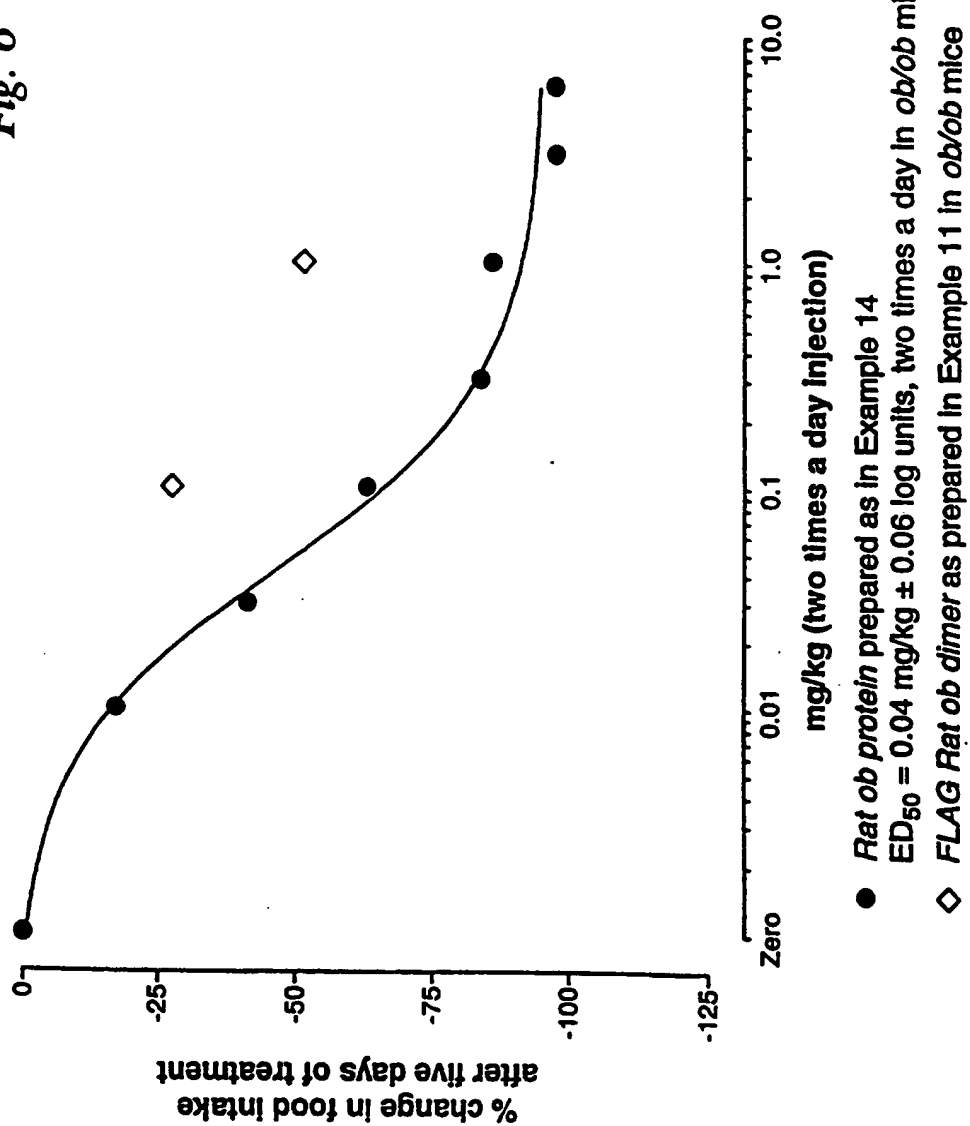
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Fig. 5



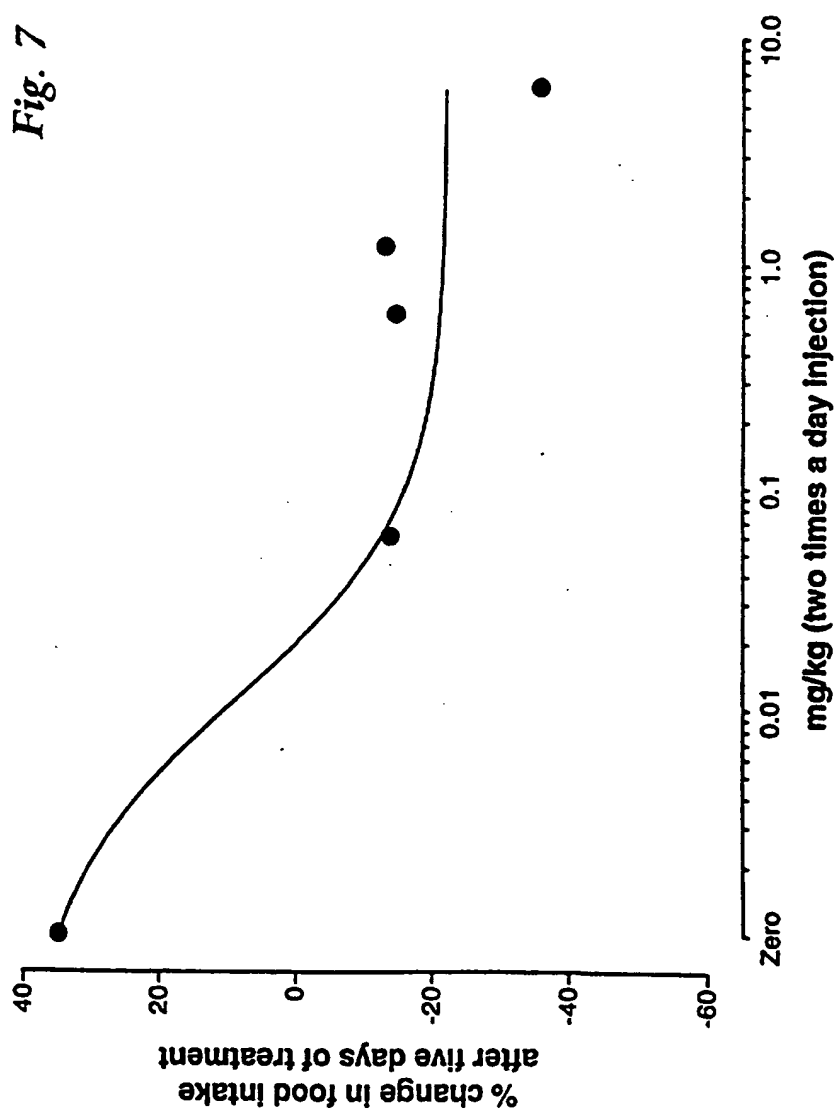
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Fig. 6



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Fig. 7

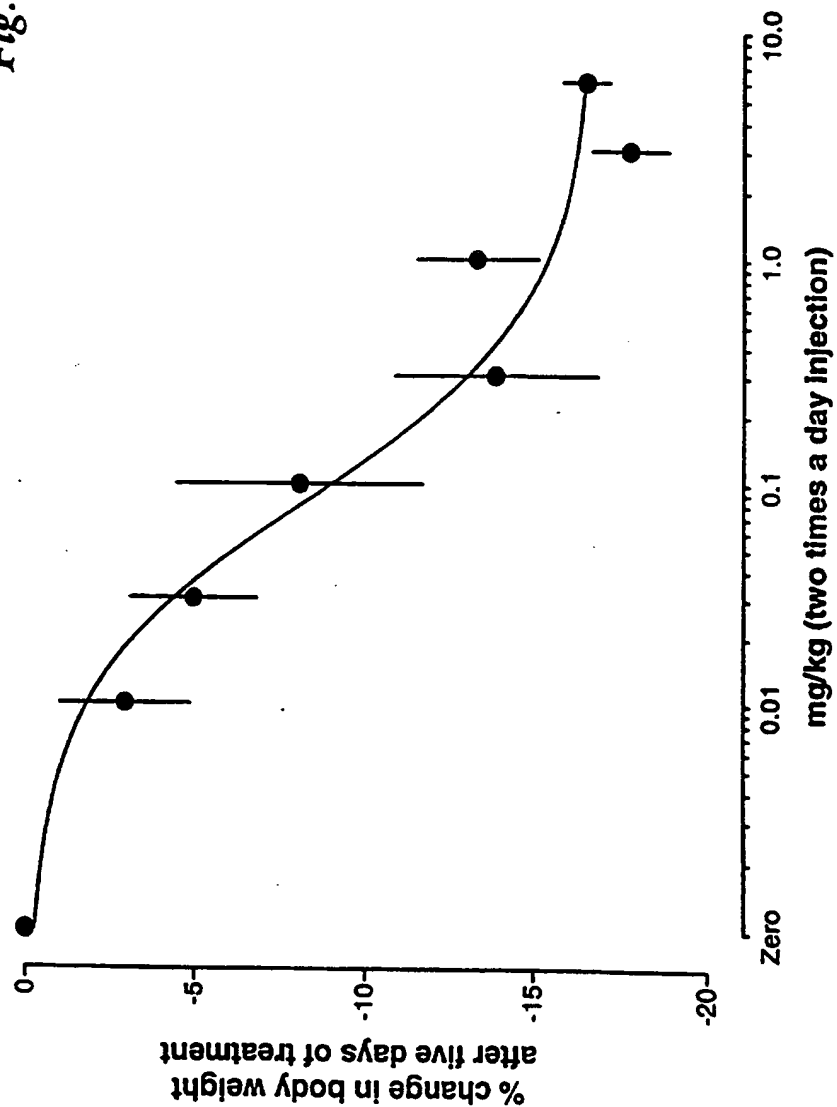


- Rat ob protein prepared as in Example 14  
ED<sub>50</sub> = 0.01 mg/kg  $\pm$  0.87 log units, two times a day in NIH/Sw mice



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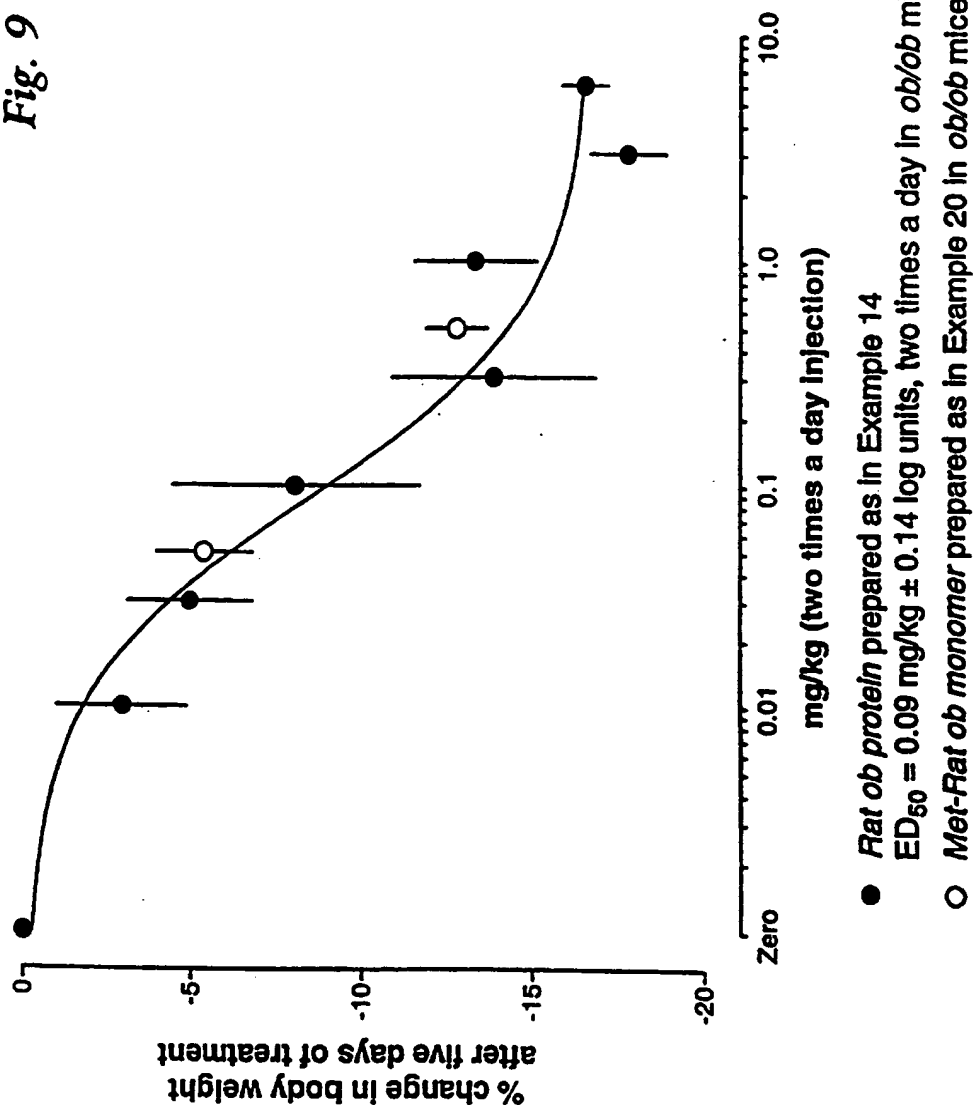
Fig. 8



● Rat ob protein prepared as in Example 14  
ED<sub>50</sub> = 0.09 mg/kg  $\pm$  0.14 log units, two times a day in ob/ob mice

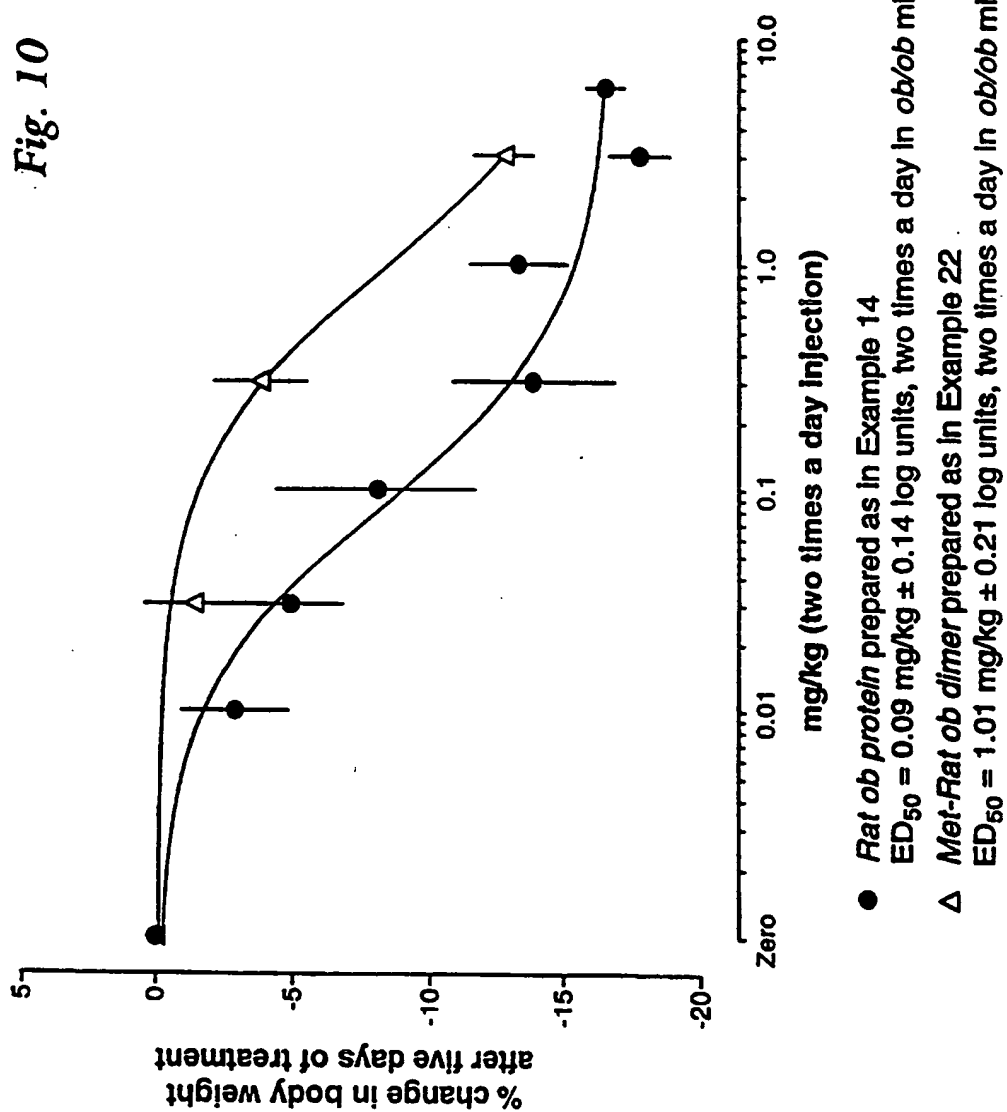
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Fig. 9



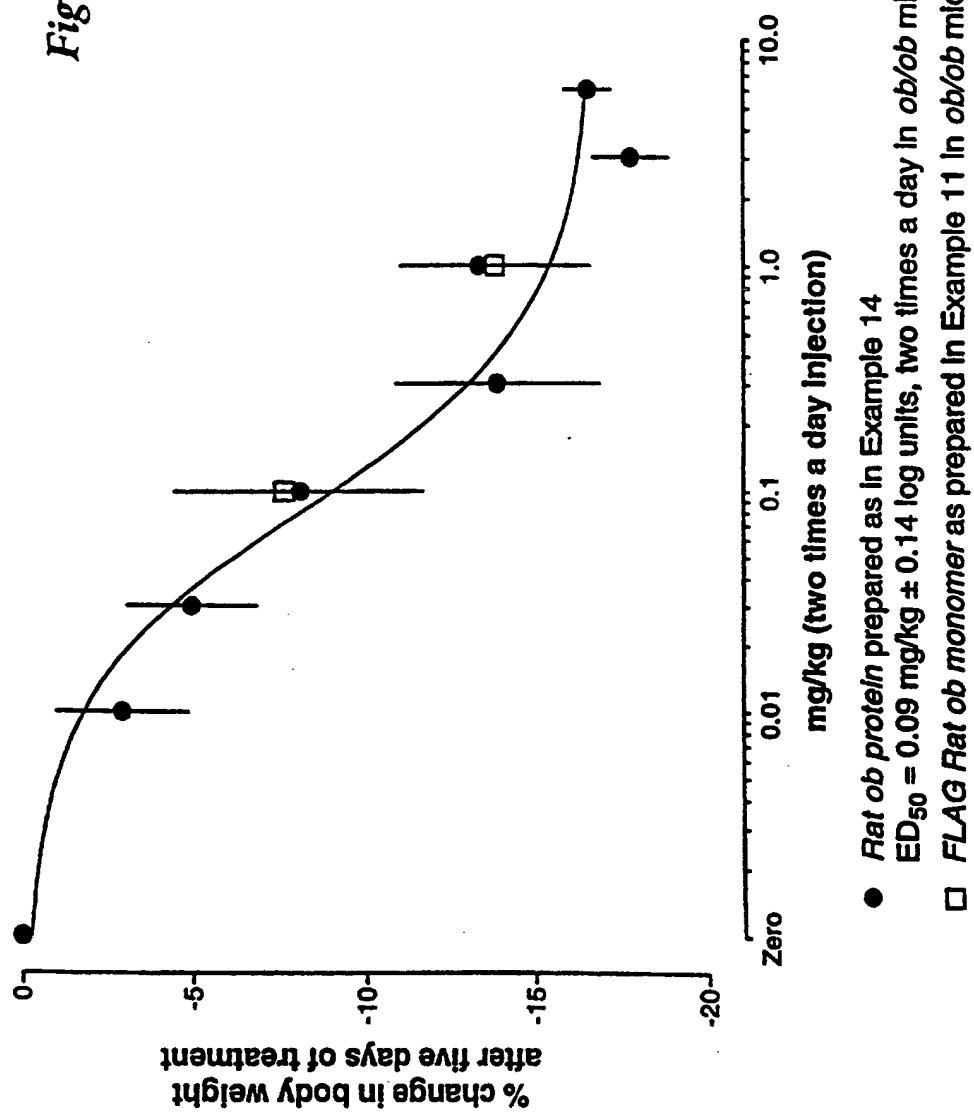
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Fig. 10



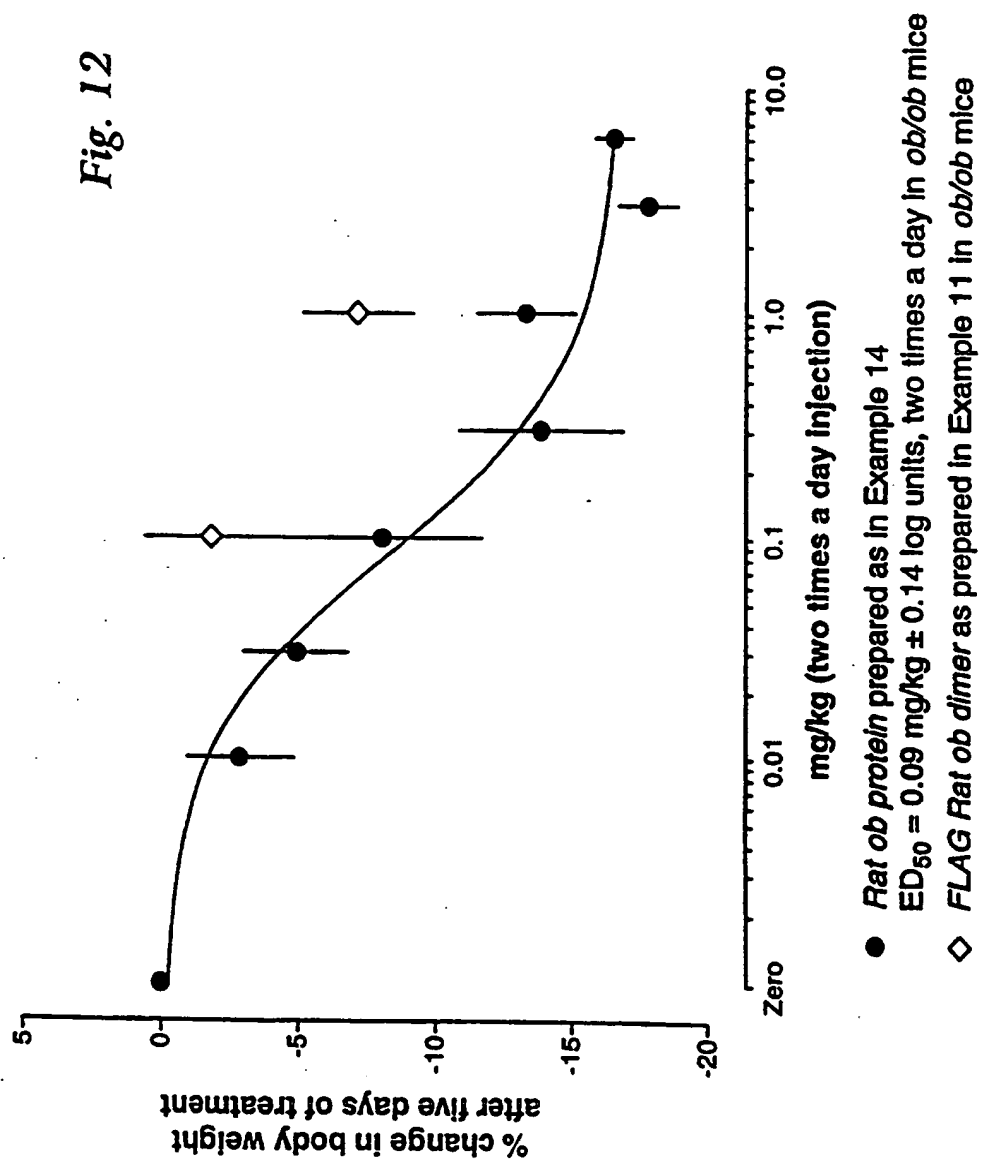
11/13

Fig. 11



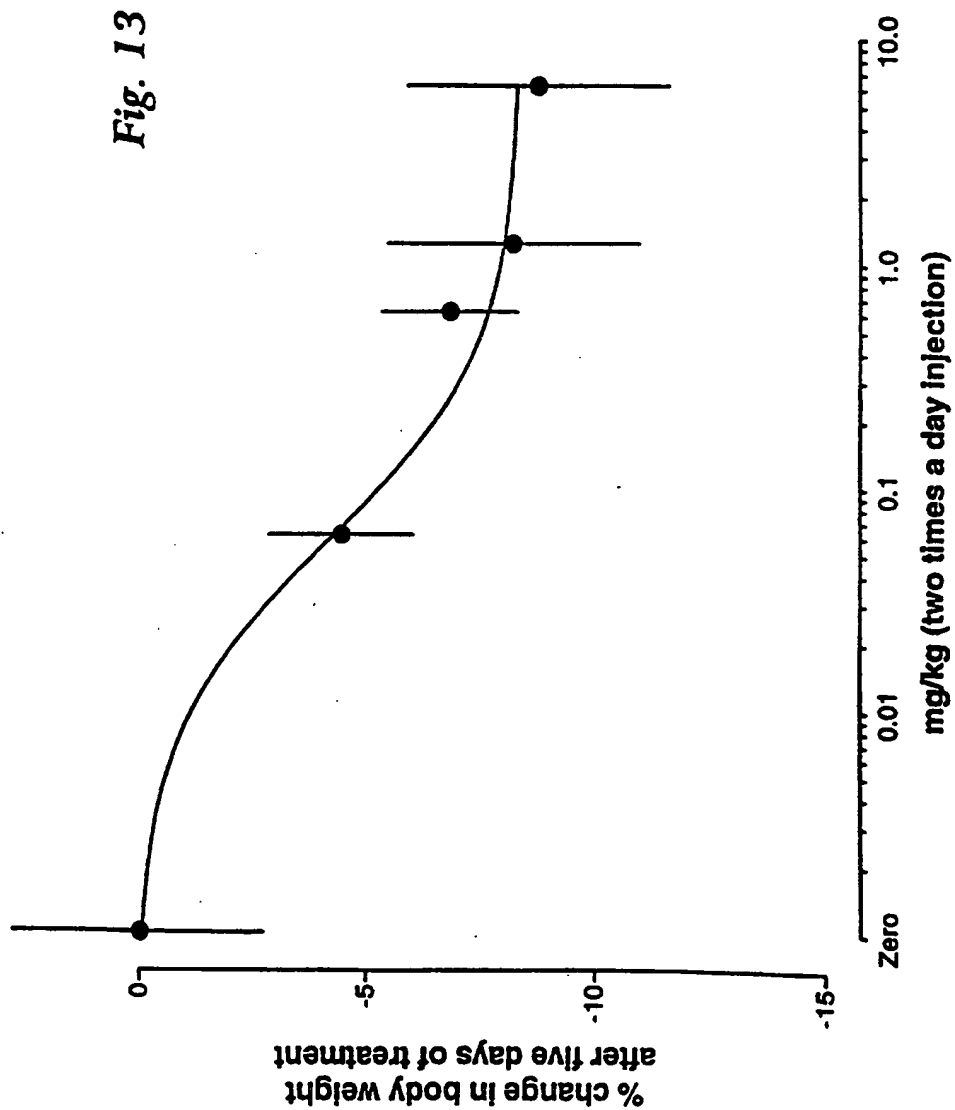
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Fig. 12



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Fig. 13



- Rat ob protein prepared as in Example 14  
ED<sub>50</sub> = 0.06 mg/kg  $\pm$  0.15 log units, two times a day in NIH/Sw mice

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/04909

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350; 514/2; 536/22.1, 23.1, 24.31, 24.32; 435/6, 91, 320.1, 69.1, 240.2, 252.33

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS, EMBASE, CAPLUS, WPIDS

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y, E	US 5,521,283 A (DIMARCHI ET AL) 28 May 1996 (28/05/96), see entire document.	1-42, 74-89, 94-96
Y, E	US 5,525,705 A (DIMARCHI ET AL) 11 June 1996 (11/06/96), see entire document.	1-42, 74-89, 94-96
Y, E	US 5,532,336 A (DIMARCHI ET AL) 02 July 1996 (02/07/96), see entire document.	1-42, 74-89, 94-96
Y	ZHANG et al. Positional cloning of the mouse <i>obese</i> gene and its human homologue. Nature. 01 December 1994, Vol. 372, pages 425-432, see entire document.	1-42, 74-89, 94-96
Y, P	HE et al. The mouse <i>obese</i> gene. Journal of Biological Chemistry. 01 December 1995, Vol. 270, No. 48, pages 28887-26891, see entire document.	1-42, 74-89, 94-96

☒ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:	*T	Inter document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z*	document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means		
*P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

09 JULY 1996

Date of mailing of the international search report

25 JUL 1996

Name and mailing address of the ISA/US  
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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/04909

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y, P	HALAAS et al. Weight-reducing effects of the plasma protein encoded by the <i>obese</i> gene. Science. 28 July 1995, Vol. 269, pages 543-546, see entire document.	1-42, 74-89, 94-96
Y, P	PELLEYMOUNTER et al. Effects of the obese gene product on body weight regulation in <i>ob/ob</i> mice. Science. 28 July 1995, Vol. 269, pages 540-543, see entire document.	1-42, 74-89, 94-96
Y, P	CAMPFIELD et al. Recombinant mouse ob protein: evidence for a peripheral signal linking adiposity and central neural networks. Science. 28 July 1995, Vol. 269, pages 546-549, see entire document.	1-42, 74-89, 94-96
Y	RINK, T.J. In search of a satiety factor. Nature. 01 December 1994, Vol. 372, pages 406-407, see entire document.	1-42, 74-89, 94-96
Y, P	STEPHENS et al. The role of neuropeptide Y in the antiobesity action of the <i>obese</i> gene product. Nature. 12 October 1995, Vol. 377, pages 530-532, see entire document.	1-42, 74-89, 94-96
Y, P	MACDOUGALD et al. Regulated expression of the obese gene product (leptin) in white adipose tissue and 3T3-L1 adipocytes. Proc. Natl. Acad. Sci. USA. September 1995, Vol. 92, pages 9034-9037, see entire document.	1-42, 74-89, 94-96
Y	WO 92/16845 A1 (AMYLIN PHARMACEUTICALS, INC.) 01 October 1992 (01/10/92), see entire document.	1-42, 74-89, 94-96
Y	WO 94/21665 A1 (AMYLIN PHARMACEUTICALS, INC.) 29 September 1994 (29/09/94), see entire document.	1-42, 74-89, 94-96



# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/04909

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:  
1-42, 74-89, and 94-96
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/04909

### A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

C07K 1/00, 14/00, 17/00; A01N 37/18, A61K 38/00; C07H 19/00, 21/00, 21/02, 21/04; C12Q 1/68; C12P 19/34, 21/06; C12N 15/00, 15/09, 15/63, 15/70, 15/74, 5/00, 1/20

### A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

530/350; 514/2; 536/22.1, 23.1, 24.31, 24.32; 435/6, 91, 320.1, 69.1, 240.2, 252.33

### BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-10, 21-24, 29, 30, 33-37, 74, 75, 78, 80, 81, and 84-89, drawn to a method of producing an ob protein as a dimer, the composition, the pharmaceutical composition, and the method of treatment using the ob dimer protein.

Group II, claims 11-20, 25-28, 31, 32, 38-42, 76, 77, 79, 82-89, and 94-96, drawn to a method of producing an ob dimer fusion protein, the composition, the pharmaceutical composition, and the method of treatment using the ob dimer fusion protein. These claims are being kept together as there is no added burden of search.

Group III, claims 43-45, 49-54, 61-64, 67, 69, 71, and 73, drawn to a monoclonal antibody which binds to an ob dimer protein and an immunoassay for detecting the presence or amount of ob dimer protein.

Group IV, claims 46-48, 55-60, 61-64, 67, 70, 72, and 73, drawn to a monoclonal antibody which binds to an ob dimer fusion protein and an immunoassay for detecting the presence or amount of ob dimer fusion protein.

Group V, claim 65, drawn to an immunoassay for detecting the presence of ob dimer protein wherein a complex is formed between ob dimer protein in the sample and antibody #1 specific for ob dimer protein and said complex is captured by antibody #2 specific for ob dimer protein and coupled to a solid carrier.

Group VI, claim 65, drawn to an immunoassay for detecting the presence of ob dimer fusion protein wherein a complex is formed between ob dimer fusion protein in the sample and antibody #1 specific for ob dimer fusion protein and said complex is captured by antibody #2 specific for ob dimer fusion protein and coupled to a solid carrier.

Group VII, claim 66, drawn to an immunoassay for detecting the presence of ob dimer protein wherein anti-ob dimer protein antibody #1 bound to a solid support is used to capture the ob dimer protein from the sample and labeled anti-ob dimer protein-specific antibody #2 is used as a developing reagent.

Group VIII, claim 66, drawn to an immunoassay for detecting the presence of ob dimer fusion protein wherein anti-ob dimer fusion protein antibody #1 bound to a solid support is used to capture the ob dimer fusion protein from the sample and labeled anti-ob dimer fusion protein-specific antibody #2 is used as a developing reagent.

Group IX, claim 68, drawn to a competition immunoassay for detecting ob dimer protein.

Group X, claim 68, drawn to a competition immunoassay for detecting ob dimer fusion protein.

Group XI, claims 90-93, drawn to the purification of ob protein.

The inventions listed as Groups I-XI do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The method of producing the ob protein as a dimer, the composition, the pharmaceutical composition, and the method of treatment using the ob dimer protein in Group I does not share a special technical feature with the method of producing the ob dimer fusion protein, the composition, the pharmaceutical composition, and the method of treatment using the ob dimer fusion protein in Group II or the purification method of Group XI. The monoclonal antibody and immunoassay method in Group III does not share a special technical feature with the monoclonal antibody and the immunoassay method of

## INTERNATIONAL SEARCH REPORT

International application No.  
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Group IV. The immunoassay method of Group V for detecting ob dimer protein does not share a special technical feature with the other immunoassay methods of Groups VII and IX for detecting ob dimer protein or the immunoassay methods of Groups VI, VIII, and X for detecting ob dimer fusion protein. Accordingly, the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.